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Remarks

Upon entry of the foregoing amendment, claims 1, 2, 4, 9-12, 14, 28, 30-44, 47-54, 62-74, 80, 81, 87, 90, 93-96, 98, 99, 102-136 are pending in this application.

Claims 3, 5-8, 13, 15-27, 29, 45, 46, 55-61, 75-79, 82-86, 88, 89, 91, 92, 97, 100 and 101 have been canceled without prejudice or disclaimer. Applicants reserve the right to pursue claims directed to the canceled subject matter in a continuing or divisional application.

The election requirement previously presented has been withdrawn with respect to the species of antigen and vectors used. Therefore, only claims 62-74 stand withdrawn from examination as being directed to a non-elected invention.

Claims 1, 2, 4, 9-12, 14, 28, 30-44, 47-54, 80, 81, 87, 90, 93-96, 98, 99 and 102-136 are currently under examination. The Office erroneously included claims 55-61 under box (6) on PTOL form 326, but correctly stated pending claims on page 3 of the Office Action.

Claims 1, 2, 32-34, 37, 38, 42, 43, 47-54, 80 and 93 have been amended. The claims were amended to correct grammatical errors and to more clearly claim the invention. Claims 1, 80, 93, 102, 110, 112, 115 and 117 are the independent claims.

No new matter is believed to have been added. In view of the amendments and following remarks, reconsideration of the rejections and withdrawal thereof is respectfully requested.

Information Disclosure Statements

Applicants acknowledge with thanks the return of executed PTO 1449 forms filed January 14, 2004.

Election/Restriction requirement

Applicants acknowledge with thanks the withdrawal of the election requirement with respect to the antigen and vectors and understand the election of species of adjuvant (CpG) stands. Accordingly, claims 1, 2, 4, 9-12, 14, 28, 30-44, 47-54, 80, 81, 87, 90, 93-96, 98, 99, 102-136 are now pending and under examination as they are drawn to the species CpG as an adjuvant. Claims 62-74 remain withdrawn as being directed to a nonelected species.

Claim Objections

The Office Action at page 5 states the restriction requirement is maintained with respect to the elected species of the adjuvant CpG but withdrawn as to the vector and antigen. Applicants understand this to be withdrawal of the species election requirement of adenoviral vector and DNA encoding sequestrin. The Examiner has objected to the claims because they encompass species which are not elected (i.e., adjuvants other than CpG). Applicants respectfully traverse the objection.

The Office noted on page 5 that in the instant case no generic nor linking claim has been found to be allowable. As in the previous Office Action, Applicants disagree and assert the generic claims herein are allowable. Applicants are not required to amend the claims to the elected species when a generic claim is allowable. Withdrawal of the objections is respectfully requested.

Rejection under 35 U.S.C. § 112, first paragraph

Applicants acknowledge with thanks the withdrawal of the rejection of claims 1, 2, 4, 9-12, 14, 28, 30-44, 47-54, 80, 81, 87, 90, 93-96, 98, 99, 102-127 under 35 U.S.C. § 112, first paragraph.

Priority

At pages 4-5, the Office continues to deny the instant application benefit of the filing date of the earliest priority document. The Office argues review of the priority documents provides no

literal or figurative support for the delivery of DNA vectors; that review of the priority documents provides teaching only for molecules that are antigens themselves, not for antigens that are produced by DNA vectors; and, that there is no literal or figurative support in the priority applications that the use of CpG was contemplated. The Office concludes the priority documents for the instant application fail to adequately support DNA vaccines and the methods and consideration of using DNA vaccines. Applicants strongly disagree with the conclusion and request that the application be granted the benefit of at least the July 17, 1997 priority date.

The generic claims are entitled to a priority date of at least July 17, 1997, the filing date of USPN 5,980,898 ('898) (See, column 14, lines 13-29, for example) as previously explained. Column 14, lines 13-29, of the '898 patent discloses, for example, that the "nucleic acid(s) contained in the formulation may encode the antigen, the adjuvant or both." Column 14, lines 13-29, further discloses that the "nucleic acid may further comprise regulatory regions operably linked to the sequence encoding the antigen or adjuvant" and that the "nucleic acid may comprise regions derived from viral genomes." Thus, the '898 patent specifically discloses use of nucleic acids encoding antigens and comprising regions derived from viral genomes in the process of immunization. In addition, the '898 patent also discloses use of bacterial DNA as an adjuvant (See, column 11, line 37: "bacterial DNA (Stacey *et al.,* 1996)"). Thus, contrary to the allegations of the Office, the '898 patent (priority U.S. Appl. No. 08/896,085, filed July 17, 1997) clearly provides literal support that the use of bacterial DNA as an adjuvant was contemplated. Applicants strongly assert the priority documents for the instant application adequately provide support for both DNA vaccines and the methods of using DNA vaccines employing bacterial DNA.

Use of bacterial DNA as an adjuvant was known in the art before the earliest priority date claimed by the instant application. Use of nucleic acid having immunostimulatory properties and which contained specifically identified CpG motifs demonstrated to confer immunostimulation were preferred for use prior to the applicable filing date. In 1996, Stacey *et al.* (1996)

[Document YR on PTO form 1449, filed Dec. 29, 1999) (Stacey *et al.*, J. Immunology 157: 2116 (1996))[copy attached as Exhibit A] disclosed that bacterial DNA can activate immune cells and that work suggests that structures or sequences in bacterial DNA that do not exist in mammalian DNA are recognized by the immune system (page 2116, left column, first paragraph, lines 8-11). Stacey disclosed (page 2120, left column, last paragraph, lines 3-4) that others in the field obtained similar results (citing Krieg *et al.*, "Krieg (4) found that bacterial DNA induced proliferation and IgM production by B cells and that this was dependent on unmethylated CpG motifs in the bacterial DNA"). Stacey and others (citing Halpern *et al.* (26) published 1996; page 2120, right column, second full paragraph, lines 2-3) found that bacterial DNA was a more effective activating agent than oligonucleotide.

Post-priority date work also demonstrated the role of bacterial DNA in the activation of the immune response. In 1998, Krieg *et al.* (Krieg *et al.*, PNAS 95: 12631 (October 1998) [copy attached as Exhibit B] demonstrated prokaryotic DNA contributed to the immunogenicity of DNA vaccines (See, for example, page 12635, left column, second full paragraph). In fact, the ability of unmethylated CpG to stimulate an immune response was so well recognized in the art that in 1998, Sun *et al.*, stated in the Journal of Experimental Medicine (JEM 187(7):1145 (April 1998))[copy attached as Exhibit C] that

> "It is now well established that unmethylated CpG dinucleotide motifs of bacterial DNA have the capacity to cause polyclonal activation of B cells and stimulation of APCs (citing references 1-8). The immunostimulatory properties of unmethylated CpG motifs is not unique to bacteria and applies to a wide spectrum of non-vertebrates includes, insects, nematodes, mollusks and yeast."

Thus, the immunostimulatory effects of CpG motifs were known in the art at least from before the priority date of the earliest claimed application. The Office is reminded that Applicants are not required to disclose what is known in the art. Clearly, use of bacterial DNA as an adjuvant

was established before the date of the priority document (July 17, 1997). Applicants assert the instant application should be accorded the filing date of the priority document (July 17, 1997) and an interference declared.

Rejection under 35 USC § 102(e) over Tang *et al.* (USPN 6,348,450)

At page 7 of the Office Action, the Office rejected claims 1, 2, 4, 9-12, 14, 28, 30-44, 47-54, 80, 81, 87, 90, 93-96, 98, 99 and 102-136 under 35 USC § 102(e) as being anticipated by Tang *et al.* (USPN 6,348,450). The rejection is respectfully traversed.

Applicants' arguments above regarding the priority date are pertinent here and are incorporated herein in their entirety. Tang is not prior art to instant claims 1, 2, 4, 9-12, 14, 28, 30-44, 47-54, 80, 81, 87, 90, 93-96, 98, 99 for reasons discussed above regarding the priority dates. Reconsideration and withdrawal of the rejection is respectfully requested.

Regarding claims 102-116, claims 102-116 were previously added to provoke an interference under 37 CFR § 1.607 (Amendment filed July 10, 2001). As previously discussed in the July 2001 amendment (and in this Reply, below) claims 102-116 represent copies of claims 1-15 of the Khavari patent. The differences in claim language are inconsequential modifications ("organism" instead of "vertebrate", "penetration enhancer" instead of "irritant"). Applicants note with interest that the Office has therefore, essentially, cited anticipatory prior art (Tang, an issued US patent) against Khavari, another issued U.S. patent.

Claims 117-127 were previously (amendment of March 14, 2002) added to provoke an interference with the Tang patent. As previously discussed in the March 2002 amendment (and again, below), claims 117-127 represent copies of, *inter alia*, Tang patent claims 1-3, 16-19, 24-29, 35-39, 44-47 and 52. The differences in claim language are inconsequential modifications (for example, "organism" instead of "mammal," "polynucleotide expressing antigen, adjuvant or both with an operably linked regulatory region derived from a viral genome" instead of a "DNA

viral vector which encodes a gene of interest") so as to accommodate the differences between the instant specification and the patent. The Office has therefore just cited Tang against Tang's own claims.

In view of the arguments above, reconsideration and withdrawal of the rejection is respectfully requested.

Rejections under 35 USC § 102(e) over Kreig *et al.* (USPN 6,339,068)

At page 7 of the Office Action, the Office rejected claims 1, 2, 4, 14, 28, 30-44, 47-54, 80, 81, 87, 90, 93, 94, 96, 98, 99 and 102-136 under 35 USC § 102(e) as being anticipated by Kreig *et al.* (USPN 6,339,068). The rejection is respectfully traversed.

Applicants' arguments above regarding the priority date of the instant application are pertinent here and are incorporated herein in their entirety. The Office alleges that Krieg teaches methods and materials for immunization protocols for the delivery and expression of polynucleotide vectors; that Krieg details the use of CpG sequences as adjuvants, that transdermal delivery can be used (specifically citing to column 10, lines 34-60) and, that Krieg provides detailed guidance on specific vectors, promoters and various formulations for the delivery of DNA vaccines. However, Krieg does not anticipate the instant invention as claimed. It is well settled that for a reference to be anticipatory, a reference must teach each and every element of the claim. The passage cited by the Office (column 10, lines 34-60) does not teach a method for inducing an antigen-specific immune response comprising, *inter alia*, applying a formulation epicutaneously to the skin of an organism without passing through the dermis of the skin. Since Krieg does not teach each and every element of independent claims 1 and 80, Krieg does not and cannot anticipate claims 1, 80 and claims dependent therefrom. If the Office maintains this rejection, the Office is respectfully requested to point out with particularity where Krieg teaches epicutaneous administration to skin of an organism.

Regarding independent claim 93, Krieg does not anticipate claim 93 and claims dependent

therefrom for the same reasons Krieg does not anticipate claims 1 and 80. Krieg fails to teach each and every element of independent claim 93. Claim 93 is directed to a formulation, which comprises, *inter alia*, an adjuvant which enhances an antigenic immune response wherein the enhancement of the immune response by the adjuvant is separable from the antigen specific immune response induced by an immunogenic epitope of the antigen. As above, Krieg does not teach each and every element of claim 93 and therefore does not, and cannot, anticipate independent claim 93 and claims dependent therefrom. If the Office maintains this rejection, the Office is respectfully requested to point out with particularity where Krieg teaches "an adjuvant which enhances an antigenic immune response wherein the enhancement of the immune response by the adjuvant is separable from the antigen specific immune response induced by an immunogenic epitope of the antigen."

Regarding claims 102-116, claims 102-116 were previously added to provoke an interference under 37 CFR § 1.607 (Amendment filed July 10, 2001). As previously discussed in the July 2001 amendment (and in this Reply, below) claims 102-116 represent copies of claims 1-15 of the Khavari patent. The differences in claim language are inconsequential modifications ("organism" instead of "vertebrate", "penetration enhancer" instead of "irritant"). The Office has, essentially, just cited anticipatory prior art (Krieg, an issued US patent) against the claims of the Khavari patent, another issued U.S. patent. Therefore, the Office has stated on the record that the Khavari patent is unpatentable over the Krieg patent.

Regarding claims 117-127, claims 117-127 were previously (amendment of March 14, 2002) added to provoke an interference with the Tang patent. As previously discussed in the March 2002 amendment (and again, below), claims 117-127 represent copies of, *inter alia*, Tang patent claims 1-3, 16-19, 24-29, 35-39, 44-47 and 52. The differences in claim language are inconsequential modifications (for example, "organism" instead of "mammal," "polynucleotide expressing antigen, adjuvant or both with an operably linked regulatory region derived from a viral genome" instead of a "DNA viral vector which encodes a gene of interest") so as to

accommodate the differences between the instant specification and the patent. Applicants note with interest that the Office has cited Krieg, an issued US patent, against the claims of the issued Tang patent. The Office therefore has admitted on the record that Tang is unpatenable over Krieg.

In view of the arguments above, reconsideration and withdrawal of the rejections is respectfully requested.

Rejection under 35 USC § 103 over Khavari *et al.* in view of Krieg *et al.* (USPN 6,339,068)
At page 8 of the Office Action, the Office rejected claims 1, 2, 4, 9-14, 19-26, 28, 30-44, 47-77, 80, 81, 87, 90, 93-96, 98, 99 and 102-136 under 35 USC § 103 as being unpatentable over Khavari *et al.* (USPN 6,087,341) in view of Krieg *et al.* (USPN 6,339,068). The rejection is respectfully traversed.

Regarding claims 1, 2, 4, 9-14, 19-26, 28, 30-44, 47-77, 80, 81, 87, 90, 93-96, 98 and 99, Applicants' arguments above regarding the priority date of the instant application and Khavari *et al.* are pertinent and are incorporated herein in their entirety. Khavari is not prior art to the instant claims for reasons discussed above. Therefore Khavari fails as the primary reference. The arguments above concerning Krieg are pertinent and are also incorporated herein in their entirety. Briefly, Krieg does not teach a method for inducing an antigen-specific immune response comprising, *inter alia*, applying a formulation epicutaneously to the skin of an organism without passing through the dermis of the skin. Since Krieg is silent on a method for inducing an immune response comprising applying a formulation as is claimed, Krieg can not render obvious the claimed invention. Reconsideration and withdrawal of the rejection is respectfully requested.

Regarding claims 102-116 (claims corresponding to the "Khavari claims"), and 117-127 (claims corresponding to the "Tang claims"), the Office has cited Krieg (an issued US patent) against the

claims corresponding to both the Khavari patent and the Tang patent. Therefore, the Office has essentially stated on the record that both the Khavari and Tang patents are obvious and unpatentable over Krieg.

Interference

As discussed above, the instant application is entitled to a priority date of at least July 17, 1997. Since the claims herein are allowable, Applicants again request the declaration of interference with Khavari (USPN 6,087,341) and an interference with Tang *et al.* (USPN 6,348,450).

Khavari has a filing date of Feb. 12, 1998, approximately 7 months after the effective filing date of the instant application (July 17, 1997). Claims 102-116 were previously added to provoke an interference under 37 CFR § 1.607 (Amendment filed July 10, 2001). As previously discussed in the July 2001 amendment, claims 102-116 represent copies of claims 1-15 of the Khavari patent. The differences in claim language are inconsequential modifications ("organism" instead of "vertebrate", "penetration enhancer" instead of "irritant").

It is believed an appropriate interference count would be claim 102 of the instant application and Khavari claim 1. Thus, Khavari claims 1-16 and Applicants' claims 102-116 are directed to the same invention.

Applicants have also previously requested an interference also be declared with USPN 6,348,450 (Tang *et al.*) in the amendments filed January 14, 2004 and March 14, 2002. Tang has a priority of August 13, 1997, approximately one month after the effective filing date of the instant application (July 17, 1997). Claims 117-127 were previously (amendment of March 14, 2002) added to provoke an interference with the Tang patent. As previously discussed in the March 2002 amendment, claims 117-127 represent copies of, *inter alia*, Tang patent claims 1-3, 16-19, 24-29, 35-39, 44-47 and 52. The differences in claim language are inconsequential modifications (for example, "organism" instead of "mammal," "polynucleotide expressing

antigen, adjuvant or both with an operably linked regulatory region derived from a viral genome" instead of a "DNA viral vector which encodes a gene of interest") so as to accommodate the differences between the instant specification and the patent.

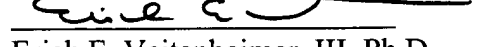
It is believed an appropriate interference count would be Tang patent claim 25 and Applicants' claim 117. Therefore, Tang patent claims 1-52 and Applicants' claims 117-127 would be directed to the same invention. Consideration of the request to provoke an interference under 37 CFR § 1.607 between this application and USPNs 6,348,450 (Tang) and 6,087,341 (Khavari) is respectfully requested.

Conclusion

It is believed the application is in condition for examination on the merits and such is respectfully requested. If, in the opinion of the Examiner, an interview would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the telephone number provided below.

Except for issue fees payable under 37 C.F.R. § 1.18, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 C.F.R. § 1.16 and § 1.17 which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-0310. This paragraph is intended to be a **Constructive Petition for Extension of Time** in accordance with 37 C.F.R. § 1.136(a)(3).

Respectfully submitted,
Morgan, Lewis & Bockius LLP



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Macrophages Ingest and Are Activated by Bacterial DNA[1]

Katryn J. Stacey,[2] Matthew J. Sweet, and David A. Hume[3]

Recent evidence suggests that bacterial DNA activates immune responses. Here we showed that TNF-α mRNA was induced in bone marrow-derived macrophages and the macrophage cell line RAW 264 by plasmid DNA, but not by DNaseI-digested plasmid, plasmid methylated on CpG dinucleotides, or by vertebrate genomic DNA, which is naturally largely methylated on these sequences. Synthetic polynucleotides poly d(I-C) and poly I·poly C also induced TNF-α. IL-1β and plasminogen activator inhibitor-2 mRNAs were induced by plasmid DNA, and IFN-γ-pretreated macrophages responded to DNA with induction of inducible nitric oxide synthase. The HIV-1 long terminal repeat was activated by exogenous DNA in a manner similar to TNF-α, and was also activated by a CpG-containing oligonucleotide. Transcription factor nuclear factor-κB (NF-κB) is involved in regulation of the HIV-1 long terminal repeat and many inflammatory response genes. NF-κB binding activity was increased by plasmid DNA. An important question is whether these effects involve DNA binding to a cell surface receptor that signals to the interior, or whether internalization is necessary. Here we found that plasmid was taken up by RAW 264 cells and remained sufficiently intact to code for luciferase protein. Results suggest that DNA is taken up by macrophages and characteristic bacterial DNA sequences, which include an unmethylated CpG sequence, activate a signaling cascade leading to activation of NF-κB and inflammatory gene induction. Relevance to DNA vaccination, gene therapy, antisense, and transfection studies is discussed. *The Journal of Immunology*, 1996, 157: 2116–2122.

A number of bacterial products such as LPS, lipoarabinomannan, peptidoglycan, and formyl methionine are well characterized as activators of immune function. There is increasing evidence that mammalian immune systems can distinguish between bacterial and vertebrate DNA, with bacterial DNA directly activating immune cells (1). B cell mitogenesis and total Ab production were activated by treatment with bacterial DNA but not mammalian DNA in a T cell-independent manner (2). This work suggested that structures or sequences in bacterial DNA that do not exist in mammalian DNA are being recognized by the immune system. One major difference between bacterial and vertebrate DNA is that vertebrate DNA contains a relatively low frequency of CpG dinucleotides, and those that occur tend to be methylated on the cytosine residue (3). In a study on B cells, activation by bacterial DNA required unmethylated CpG motifs, and was mimicked by some CpG-containing oligonucleotides (4). The most active oligonucleotides contained CpG flanked by two 5′ purines and two 3′ pyrimidines.

B cells are not the only cell type to respond to foreign DNA. Using murine spleen cells, Yamamoto et al. (5) showed that viral, bacterial, and invertebrate DNA increased NK cell activity and induced IFNs, whereas plant and vertebrate DNA did not. This activation correlates well with the DNA methylation pattern, only vertebrate and plant DNAs are largely methylated on CpG motifs (3, 6, 7). CpG-containing palindromic sequences from bacterial DNA were identified that induced NK cell activity (8). Some of these sequences are similar to those found by Krieg et al. (4) for B cell activation. Injection of these CpG-containing oligonucleotides or bacterial DNA into tumors has been found to lead to immune cell infiltration and tumor regression (9).

Published evidence suggests that entry of DNA into the cell is required for activation. Immobilized oligonucleotide was unable to stimulate B cells (4), and lipofection of active oligonucleotides to increase cellular uptake greatly enhanced their effect in activating NK activity (10). Also, no difference has been detected in the binding of activating and nonactivating oligonucleotides to the cell surface (4, 10). This suggests that there is no discrimination at the level of cell surface binding, and determination of whether DNA is of foreign origin may take place intracellularly. Uptake of DNA by monocytes has been observed, but it was largely degraded within an endosomal compartment (11). In this paper we show that some DNA taken up into a macrophage cell line remains in an intact form and can be expressed, and that bacterial DNA activates transcription factor nuclear factor-κB (NF-κB),[4] and expression of a number of genes in macrophages. Cellular responses to DNA have experimental implications for transfection and antisense oligonucleotide studies, and therapeutic implications in gene therapy, DNA immunization, and antisense therapy.

Materials and Methods

Materials

The synthetic dsDNA, poly d(I-C), was purchased from Boehringer Mannheim. Salmon sperm DNA, poly I·poly C (dsRNA), poly dC (ssDNA), and poly I and poly C (ssRNAs) were purchased from Sigma Chemical Co. (St. Louis, MO). LPS from *Salmonella minnesota* RE595 and *Escherichia coli* serotype 0111:B4 were purchased from Sigma Chemical Co. Murine IFN-γ was purchased from Genzyme (Cambridge, MA).

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[4] Abbreviations used in this paper: NF-κB, nuclear factor-κB; BMM, bone marrow-derived macrophages; PAI-2, plasminogen activator inhibitor-2; LTR, long terminal repeat; EMSA, electromobility shift analysis.

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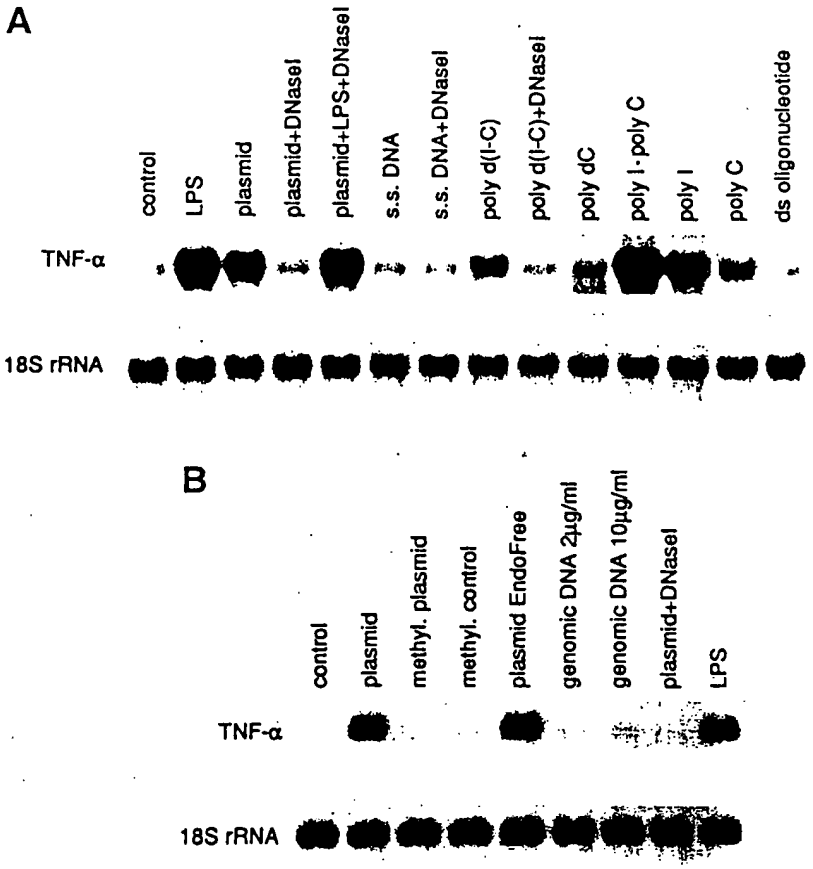

FIGURE 1. Northern blot analysis of induction of TNF-α mRNA by nucleic acids. *A*, BMM were incubated in 10 ml medium for 1 h with LPS or various nucleic acids. Treatments were: control, no additions; LPS, 10 ng *Salmonella minnesota* LPS; plasmid, 10 μg pBluescript; plasmid + DNaseI, 10 μg pBluescript DNA digested with DNaseI; plasmid + LPS + DNaseI, 10 μg pBluescript DNA and 10 ng *S. minnesota* LPS treated with DNaseI; s.s. DNA, 10 μg salmon sperm DNA; s.s. DNA + DNaseI, 10 μg salmon sperm DNA digested with DNaseI; 10 μg poly d(I-C); 10 μg poly d(I-C) digested with DNaseI; 10 μg poly dC; 10 μg poly I-poly C; 10 μg poly I; 10 μg poly C; ds oligonucleotide, 10 μg ds oligonucleotide (5′-GGCCAGGACCAAATG AGGAGATCTT-3′). Hybridization to 18S rRNA is shown as a loading control. *B*, RAW 264 cells were incubated in 5 ml medium for 1 h with LPS or various nucleic acids. Cell treatments were: control, no additions; plasmid, 10 μg pBluescript; methyl. plasmid, 10 μg pBluescript treated with CpG methylase; methyl. control, CpG methylase in buffer for methylation; plasmid EndoFree, 10 μg pBluescript prepared with Qiagen EndoFree kit; genomic DNA 2 μg/ml, 10 μg mouse genomic DNA; genomic DNA 10 μg/ml, 50 μg mouse genomic DNA; plasmid + DNaseI, 10 μg pBluescript digested with DNaseI; LPS, 10 ng/ml *E. coli* LPS. Hybridization to 18S rRNA is shown as a loading control.

Cell culture

The murine macrophage-like cell line RAW 264 (12) was obtained from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 with 10% FCS, 20 U/ml penicillin, and 20 μg/ml streptomycin. Bone marrow-derived macrophages (BMM) were obtained from BALB/c mice as described (13) and used after 7 days in culture.

Northern Blot analysis

Cells were plated on tissue culture plastic approximately 16 h before commencement of the experiment. Nucleic acids or LPS were added to the cells for various times as indicated in the figure legends. RNA was prepared by the method of Chomczynski and Sacchi (14). Ten micrograms of each sample was run on denaturing MOPS formaldehyde gels, blotted, and probed according to Hybond N protocols (Amersham, Arlington Heights, IL). cDNA probes were labeled with [α-^{32}P]dCTP by random priming (Amersham). An oligonucleotide probe complementary to mouse 18S rRNA (5′-CATGGTAGGCACGGCGACTACCAT-3′) was used as a loading control, and was end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase.

DNA preparation

pBluescriptSK (Stratagene, La Jolla, CA) and pGL3C (Promega, Madison, WI) were prepared by alkaline lysis followed by CsCl gradient centrifugation. Ethidium bromide was then removed by repeated extraction with isopropanol, plasmid was diluted threefold, and precipitated with 2 vol of ethanol. The precipitation was repeated and the pellet rinsed with 70% ethanol before resuspension in water. Mouse genomic DNA prepared by the method of Laird et al. (15) was obtained from Dr. R. Passey (Heart Research Institute, Sydney, Australia). This DNA was then treated with RNaseA, followed by phenol/chloroform extraction, ether extraction, and ethanol precipitation. For digestion of DNA with DNaseI, 20 to 50 μg of DNA was incubated with 10 U of RNase-free DNaseI (Boehringer Mannheim, Mannheim, Germany) in 20 mM Tris-HCl, pH 7.6, and 20 mM $MgCl_2$ for 6 to 8 h. Complete digestion was confirmed by agarose gel electrophoresis. For methylation of plasmid DNA, 30 μg of pBluescriptSK was incubated with 15 U CpG methylase (New England Biolabs, Beverly, MA) and 160 μM *S*-adenosylmethionine in 10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT. Methylation was complete, as assessed by inability of the methylation-sensitive enzyme *Hpa*II to digest the methylated plasmid DNA. Single stranded oligonucleotides used were AAC-22 (5′-ACCGATAACGTTGCCGGTGACG-3′) and ACC-22 (5′-ACCGATACCGGTGCCGGTGACG-3′) (10).

Activation of A4 cells

A4 cells, which are RAW 264 cells stably transfected to express the luciferase gene under the control of the HIV-1 long terminal repeat (LTR), have been described previously (16). Cells were plated out 16 h before each experiment (10^6 cells in 5 ml medium). Medium was reduced to 1 ml and then LPS or nucleic acid was added to the medium for 2 h. Cells were harvested for luciferase analysis per Promega protocols.

Electromobility shift analysis (EMSA) of NF-κB activity

BMM were plated out overnight in medium with 10^4 U/ml CSF-1, and then DNA or LPS was added for 1 h before harvest of cells. Nuclear extracts were prepared as described previously (17), except that 0.5% Nonidet P-40 was used in the cell lysis. Two micrograms of nuclear protein extract was incubated with 0.02 pmol of end-labeled double stranded oligonucleotide probe in a total volume of 10 μl, containing 20 mM HEPES (pH 7.9), 0.5 mM DTT, 12% glycerol, 40 mM KCl, 0.5 μg poly d(I-C), and 1 mM EDTA. Samples were incubated at room temperature for 30 min, and then run on 5% acrylamide gel (acrylamide:bis ratio, 29:1) at 100 V in 1× Tris-borate-EDTA buffer. Gels were dried and exposed to x-ray film. The double stranded oligonucleotide probe used is a NF-κB binding site from the TNF-α promoter (κB site 3 (18) - 5′-CAA ACA GGG GGC TTT CCC TCC TC-3′).

Results

Treatment of both BMM cells and the mouse macrophage cell line RAW 264 with pBluescript plasmid DNA (1 or 2 μg/ml) for 1 h led to induction of TNF-α mRNA (Fig. 1, *A* and *B*). Since TNF-α is well characterized as an LPS-inducible gene, it was necessary to

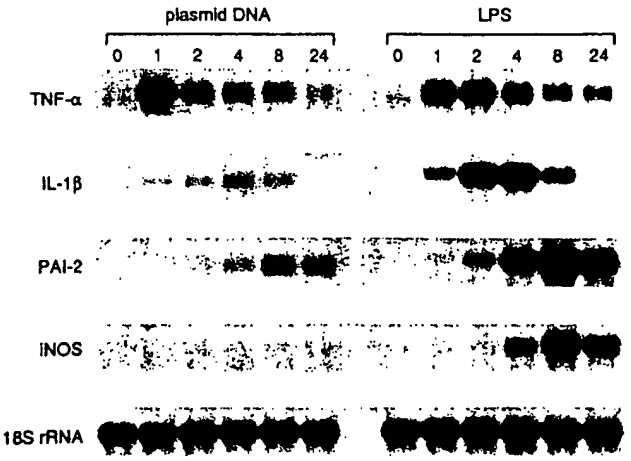

FIGURE 2. Northern blot analysis of the timecourse of induction of various genes by LPS and plasmid DNA. RAW 264 cells were incubated from 1 to 24 h with either 100 ng/ml *Salmonella minnesota* LPS or 2 μg/ml pBluescript. Expression of TNF-α, IL-1β, PAI-2, and iNOS mRNA was assessed by hybridization with labeled cDNA probes. Hybridization to 18S rRNA is shown as a loading control.

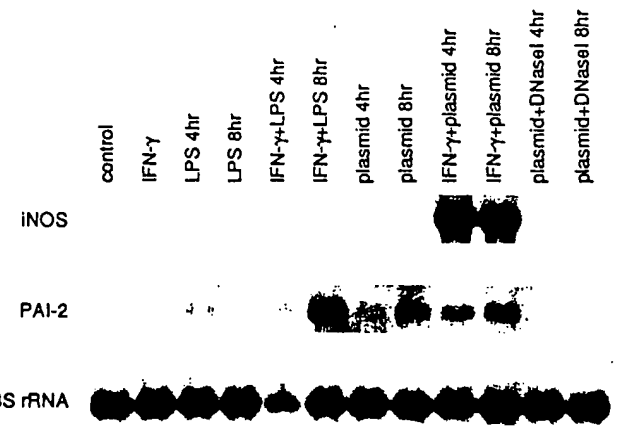

FIGURE 3. Northern blot analysis of the effect of IFN-γ pretreatment on induction of PAI-2 and iNOS mRNA. RAW 264 cells were plated for 14 h with or without 20 U/ml murine IFN-γ. Control and IFN-γ samples were harvested at this time, and other samples were treated for a further 4 or 8 h with either 0.1 ng/ml *Salmonella minnesota* LPS, 5 μg/ml pBluescript, or DNaseI-treated plasmid. The concentration of LPS used was suboptimal, to best see the priming effect of IFN-γ. Expression of PAI-2 and iNOS mRNA was assessed by hybridization with labeled cDNA probes. Hybridization to 18S rRNA is shown as a loading control.

determine whether this induction was due to contaminating LPS. Digestion of the plasmid DNA with DNaseI prevented any response (Fig. 1, *A* and *B*), showing that the DNA itself was responsible for the induction of TNF-α. If plasmid DNA was spiked with LPS before DNaseI treatment, the response to LPS was unchanged (Fig. 1*A*), showing that the DNaseI treatment in no way diminished the response to LPS. In addition, plasmid prepared by a method designed to remove endotoxin (EndoFree; Qiagen, Hilden, Germany) gave the same level of TNF-α induction (Fig. 1*B*). Induction of TNF-α by plasmid DNA led to the question of what other forms of DNA could produce a similar response. Figure 1*A* shows that the synthetic dsDNA poly d(I-C) also induced TNF-α, and this effect was prevented by DNaseI digestion. In other experiments (data not shown), the level of induction by poly d(I-C) was similar to that by plasmid. Single stranded poly dC did not induce TNF-α message (Fig. 1*A*). Some background hybridization in this lane was due to probe hybridization to poly dC taken up by or bound by the cells at the time of harvest. (The TNF-α cDNA used as probe contains poly dG sequences introduced during cloning.) Although not necessarily acting via the same pathway as DNA, synthetic RNAs also induced TNF-α mRNA. Synthetic dsRNA (poly I·poly C) was the most active, followed by poly I and poly C (Fig. 1*A*). However, salmon sperm DNA (Fig. 1*A*) and mouse genomic DNA (Fig. 1*B*) were without effect on TNF-α mRNA levels. This lack of effect is likely to be due to the methylation of vertebrate DNA, as TNF-α was no longer induced when plasmid was methylated on CpG motifs (Fig. 1*B*). A double stranded oligonucleotide which contained no CpG sequence was also nonstimulatory (Fig. 1*A*).

Since DNA is able to mimic the effect of LPS in induction of TNF-α, its effect on the induction of a number of other LPS-inducible genes was investigated. Figure 2 shows timecourses of treatment of RAW 264 cells with either LPS or plasmid DNA. TNF-α was strongly induced by a 1-h treatment with DNA, and induction was a little less sustained than in response to LPS. IL-1β, an important inflammatory mediator in response to LPS (19), was induced with similar kinetics by LPS and DNA, but the DNA response was lower. Plasminogen activator inhibitor type 2 (PAI-2) is another LPS-responsive macrophage gene (20), and was induced by DNA. Treatment of plasmid with DNaseI prevented the induction of PAI-2 (Fig. 3), once again confirming that responses observed are not due to a contaminant of the plasmid. iNOS, a gene involved in the tumoricidal response of macrophages (21), was not responsive to DNA alone (Figs. 2 and 3), but was dramatically induced by DNA if the cells were pretreated with IFN-γ (Fig. 3). In contrast, IFN-γ pretreatment enhanced the induction of PAI-2 mRNA by LPS, but not by DNA (Fig. 3). Thus, despite similarities between LPS and DNA responses, the pathways of activation are not entirely convergent.

The HIV-1 LTR is another LPS-responsive promoter, and experiments were performed to assess whether it is responsive to DNA. In earlier work we had stably integrated an HIV-1 LTR-luciferase construct into RAW 264 cells, and shown the LTR to be readily activated by LPS (16). Treatment of these cells with various types of DNA and RNA showed that the HIV-1 LTR was activated by plasmid, poly d(I-C) (Fig. 4*A*), and poly I·poly C (Fig. 4*B*). No response was seen to DNaseI-digested or methylated plasmid, salmon sperm DNA, or mouse genomic DNA (Fig. 4*A*). Thus the HIV-1 LTR responded in a similar manner to the TNF-α gene. Dose-response curves for activation of the HIV-1 LTR by plasmid DNA, poly d(I-C), and poly I·poly C showed a sensitive response to concentrations of 2 μg/ml and less, with only a moderate further increase in transcription when concentration was increased from 2 to 20 μg/ml (Fig. 4*B*). Responses to plasmid concentrations as low as 0.1 μg/ml have been detected (result not shown). DNaseI-digested plasmid (Fig. 4*B*) and salmon sperm DNA (not shown) did not activate the HIV-1 LTR at concentrations of up to 20 μg/ml. Hence induction by higher concentrations of plasmid was not boosted by LPS contaminating the plasmid preparation.

Two palindrome-containing oligonucleotides that have been characterized as either activating (AAC-22) or not activating (ACC-22) NK cell activity when added as naked DNA to spleen cells (10), were also tested for ability to activate the HIV-1 LTR. AAC-22, containing the palindrome AACGTT, activated the HIV-1 LTR, whereas ACC-22, containing the palindrome ACCGGT, did not (Fig. 4*B*). No response was seen to oligonucleotide below a concentration of 2 to 5 μg/ml, so it is a less potent activator of the HIV-1 promoter than plasmid.

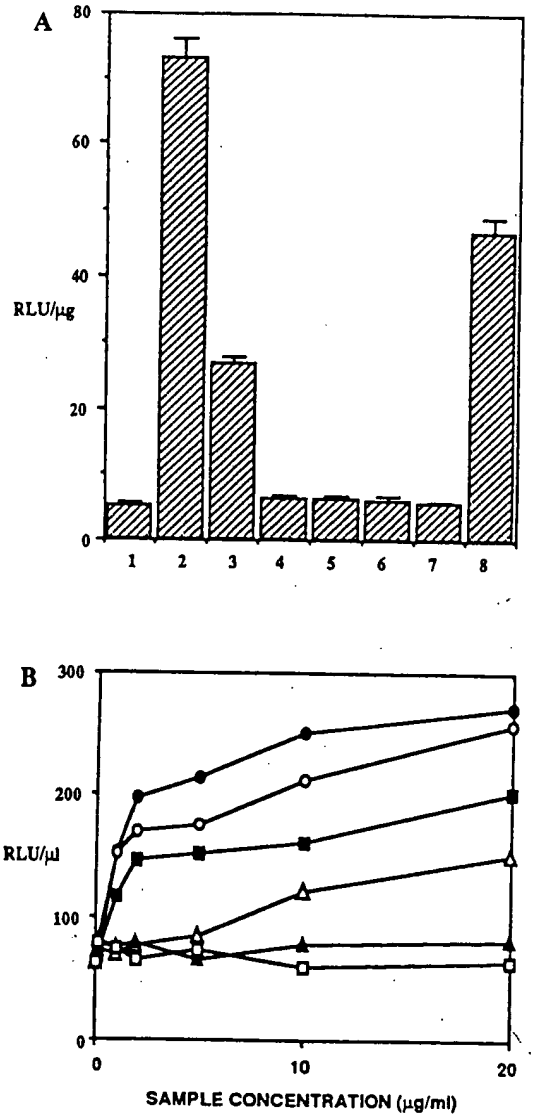

FIGURE 4. Activation of the integrated HIV-1 LTR in A4 cells by nucleic acids. *A*, A4 cells in 1 ml medium were incubated for 2 h with 1) no additions; 2) 100 ng *Salmonella minnesota* LPS; 3) 2 μg pBluescript; 4) 2 μg pBluescript digested with DNaseI; 5) 2 μg pBluescript treated with CpG methylase; 6) 2 μg salmon sperm DNA; 7) 2 μg murine genomic DNA; and 8) 2 μg poly d(I-C). Cells were then lysed and assayed for luciferase. Results are expressed as relative light units (RLU) per μg cellular protein. Error bars show the SEM (n = 5). *B*, A4 cells were incubated for 2 h with various concentrations of nucleic acids. Treatments were: poly d(I-C) (●); poly I·poly C (○); pBluescript (■); pBluescript digested with DNaseI (□); oligonucleotide AAC-22 (△); and oligonucleotide ACC-22 (▲). Cells were harvested in 100 μl lysis buffer, and results are presented as RLU/μl.

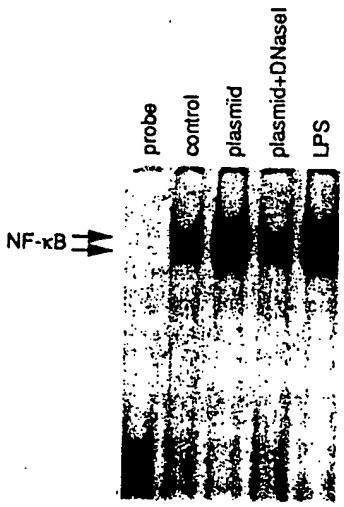

FIGURE 5. Induction of NF-κB binding activity by plasmid DNA. BMM in 10 ml medium were incubated for 1 h with the following treatments: control, no additions; plasmid, 10 μg pBluescript; plasmid + DNaseI, 10 μg pBluescript digested with DNaseI; and LPS, 100 ng/ml *Salmonella minnesota* LPS. EMSA of nuclear NF-κB was performed using an NF-κB binding site from the TNF-α promoter.

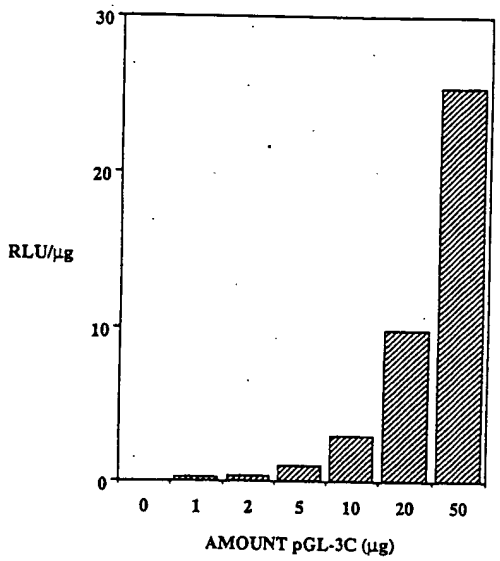

FIGURE 6. Expression of luciferase from plasmid taken up from the medium. RAW 264 cells (1.5 million) were incubated with various amounts of the luciferase reporter construct pGL3C (Promega) in 100 μl of medium for 1 h. Cells were then plated in 5 ml of medium for 23 h before harvest for analysis of luciferase activity.

The transcription factor NF-κB is activated by LPS treatment of macrophages and is implicated in induction of TNF-α, IL-1β, and HIV transcription (18, 22, 23). The possibility that NF-κB mediates some of the effects of DNA was investigated. Figure 5 shows an EMSA of BMM nuclear extracts using an NF-κB-binding oligonucleotide derived from the TNF-α promoter. In three separate experiments, treatment of cells with plasmid DNA was found to increase binding of NF-κB (Fig. 5 and unpublished observations). This increase in NF-κB was prevented by digestion of the plasmid with DNaseI (Fig. 5). CpG-methylated plasmid and salmon sperm DNA were unable to induce NF-κB binding (result not shown). Thus, the induction of NF-κB was well correlated with factors that induced TNF-α and activated the HIV-1 LTR.

An important issue in the activation of cells by exogenous DNA is whether the DNA binds a cell surface receptor and signals from outside the cell, or whether internalization is necessary. To examine whether DNA could be taken up by cells and escape from endosomes in an intact form, RAW 264 cells were incubated with various concentrations of the luciferase reporter construct pGL3C. Results in Figure 6 show that plasmid merely incubated with cells was indeed taken up and expressed in a dose-dependent manner. Cells were incubated for 1 h in a small volume with a high concentration of plasmid (10 to 500 μg/ml) and then diluted out to a

final plasmid concentration of 0.2 to 10 μg/ml for a 23-h incubation. The initial high concentration incubation was necessary to get good levels of expression, as samples incubated for 24 h with 5 μg/ml plasmid gave detectable but very low expression (result not shown).

Discussion

Work on cellular responses to DNA in our laboratory was initiated by the observation that transient transfection with DNA had an LPS-like effect on macrophages. Subsequent work showed that this cellular activation was due to exposure to bacterial DNA, and did not require electroporation. Results presented here show that treatment of macrophages with plasmid DNA induced expression of the TNF-α, IL-1β, and PAI-2 genes; induced iNOS mRNA in IFN-γ-primed cells; and activated an integrated HIV-1 LTR. Some or all of these effects may be mediated by the activation of transcription factor NF-κB, which occurred in cells exposed to plasmid DNA. NF-κB sites are found in many genes involved in the inflammatory response and NF-κB is believed to regulate TNF-α (18), IL-1β (23), and HIV-1 (22) transcription. Induction of TNF-α, NF-κB, and the HIV-1 LTR was mediated by plasmid DNA, but not salmon sperm or murine genomic DNA. This result is complementary to studies showing activation of B cells (2) and NK activity (5) by bacterial DNA, but not vertebrate genomic DNA. Thus it seems that DNA can be added to the list of bacterial products with immune-stimulating activity.

Genes or proteins reported as being induced in response to bacterial DNA are IL-6 by B cells and $CD4^+$ T cells (24, 25), IL-12 by B cells and an adherent population of spleen cells (25, 26), IFN-α/-β by spleen cell cultures (5), and TNF-α, IL-1β, PAI-2, and iNOS by macrophages (this report). Induction of TNF-α by DNA in splenocytes has previously been inferred by the finding that neutralizing anti-TNF-α Abs inhibited the induction of IFN-γ in response to DNA, although TNF-α could not be detected by ELISA (26). Our work confirms that TNF-α production in splenocyte cultures is probably due to macrophage activation. Using ELISA we could readily detect TNF-α in macrophage culture medium after addition of DNA, and in BMM, expression was enhanced by IFN-γ pretreatment (result not shown). Induction of IFN-γ by DNA has been found in NK cells and $CD4^+$ T cells (25), but this may be a secondary response to induced IL-12 and TNF-α rather than a direct effect of the DNA (25, 26). It is clear that DNA acts directly on several cell types to induce cytokines, and its action on a mixed population of immune cells is likely to result in induction of a wide range of cytokines. Here we found that treatment of macrophages with IFN-γ primed them for production of iNOS mRNA in response to DNA. Similarly, Yi et al. (24) found that IFN-γ increased B cell activation by DNA. Thus, bacterial DNA may induce cytokines such as IL-12 and TNF-α in vivo, leading to increased production of IFN-γ, which then enhances macrophage and B cell activation by DNA.

Obvious questions arising from this work are what features of bacterial DNA are recognized as foreign, and how is the recognition mediated. Krieg et al. (4) found that bacterial DNA induced proliferation and IgM production by B cells, and that this was dependent on unmethylated CpG motifs in the bacterial DNA. We have confirmed this in macrophages by showing that CpG methylation of plasmid DNA prevented it from inducing NF-κB, TNF-α, and the HIV-1 LTR. Extensive work has shown that some 6-bp palindromic sequences containing a CpG motif could induce IFN and NK cell activity in spleen cells (8, 27), and we have found that a CpG-containing palindromic oligonucleotide also activated macrophages (Fig. 4*B*). Oligonucleotide sequences mediating activation of B cells were not all palindromic, but had a preference for two 5′ purines and two 3′ pyrimidines surrounding the CpG motif (4, 25). In mammalian systems, CpG methylation and a low frequency of activating sequences may combine to prevent general immune activation by self-DNA. "CpG suppression" in vertebrates means that CpG sequences occur at approximately 25% of the level predicted from genome base composition (3). In addition to CpG suppression, Krieg et al. (4) found that B cell stimulatory hexamer sequences are found in human coding DNA sequences at less than one-third the frequency of CpG-containing hexamers with little stimulatory activity. In about 98% of the mammalian genome, approximately 70% of the CpG motifs are methylated (3). The other 2% of genomic DNA consists of unmethylated "CpG islands" associated with the 5′ ends of many genes (3). This level of CpG suppression and methylation is apparently sufficient to prevent unwanted activation of immune function. Whether there is any preferential methylation of stimulatory sequences, or a further lowered frequency of stimulatory sequences in CpG islands, has not been determined.

A number of synthetic DNA molecules such as a random copolymer, poly (dG, dC) (28), $(dG)_{18}$ (29), and in our study poly d(I-C) but not poly dC, have been shown to activate immune cells. These polynucleotides clearly do not all contain the activating sequences identified by Krieg et al. (4) and Kuramoto et al. (27), or even CpG dinucleotides. Whether they are being detected by the same system that mediates responses to bacterial DNA and active oligonucleotides is not known. It is possible that cellular activation is mediated through recognition of a DNA conformation rather than specific sequence or methylation status.

Despite the high molar concentration of stimulatory sequences when oligonucleotides are used to activate cells, we and others (26) found that bacterial DNA was a more effective activating agent than oligonucleotide. In our work with macrophages, and in spleen cell cultures (10), response to naked oligonucleotide was not detected at less than 2 μg/ml, whereas we have found responses to plasmid DNA as low as 0.1 μg/ml. We considered that this difference may be due to more rapid degradation of oligonucleotides than plasmid DNA. However, phosphorothioate-linked oligonucleotides, which are not readily degraded, were no more effective at activating macrophages, nor did using double stranded oligonucleotides have much effect (result not shown). It may be either that the combination of different stimulatory sequences found in plasmid DNA is more effective, or that longer molecules are more active. One group has found cytokine production by spleen cells in response to a 20-bp phosphorothioate-linked oligonucleotide at concentrations as low as 0.03 μg/ml (25). They also found responses to oligonucleotides of only 8 bp (4, 25), which were nonstimulatory to macrophages at even high concentrations (result not shown). Thus there may be differences in DNA detection systems between cell types, or the various responses measured may have different thresholds for activation.

Synthetic dsRNA has a well-established role in induction of IFNs and an antiviral state (30), activities that are not generally attributed to DNA (31). In this study, synthetic dsRNA and DNA had similar effects in inducing TNF-α mRNA and HIV-1 LTR activity. A candidate for mediating these responses to dsRNA is the RNA-dependent protein kinase (PKR), which is known to activate NF-κB by phosphorylating IκB (32), and may participate in tumoricidal activation of macrophages in response to LPS (33). Whether the pathways of activation by RNA and DNA converge remains to be determined. RNA certainly had additional effects on macrophages, as treatment with high concentrations of poly I·poly C was toxic, while DNA had no such effect (result not shown).

Macrophage scavenger receptors, which bind a number of polyanions, are a candidate for mediating some of the effects of nucleic acids. Poly I, and to an extent poly I·poly C, are ligands for the scavenger receptor (34, 35), and they induced TNF-α more effectively than did poly C, which does not bind to scavenger receptor. Although some of the effects seen here may be mediated through binding to scavenger receptor, it is unlikely to account for the action of all the nucleic acids tested, as phage and plasmid DNA were not found to be good ligands (35).

Most evidence suggests that determination of whether DNA is of bacterial or self-origin occurs within the cell (see introduction). A number of groups have provided evidence for specific binding of DNA to cell surface receptors (reviewed in Ref. 36). It appears that DNA uptake is receptor mediated and DNA is largely broken down within an endosomal compartment (11). While it is possible that recognition of bacterial DNA occurs within endosomes, we have shown in this paper that intact plasmid can reach the nucleus. So a protein detecting foreign DNA could also be located in the nucleus or cytoplasm. This would enable detection of viral DNA. Infection with HIV has been shown to activate NF-κB (22), and although several systems are likely to play a role in this, it could be mediated in part by detection of HIV DNA. We have previously observed cell death of primary macrophages in response to transfected DNA, and proposed that this may be a defense against viral infection (13). However, that effect and the immune activation by DNA observed here seem to be differently mediated, as transfected salmon sperm DNA was also toxic to the cells.

Cellular uptake of DNA is of interest to the fields of gene therapy and DNA immunization. For some Ags, injection of DNA encoding the Ag can lead to a protective immune response (37). It was initially shown that muscle cells at the site of injection could express β-galactosidase from injected plasmid (38), but which cell types are critical in the expression and presentation of Ag leading to an immune response has not been determined. Macrophages are a possible site of uptake and expression of the foreign DNA, and results here show that a macrophage cell line can internalize and express exogenous DNA. However, mechanisms leading to presentation of Ag in association with MHC class II molecules, which is presumably necessary for the immune response seen in DNA immunization, remain unknown. Another aspect of this work that is relevant to DNA immunization is the general activation of immune function in response to foreign DNA. The adjuvant-like activity of the DNA itself may be required for the immune response in DNA immunization.

Cellular activation by DNA has many experimental and therapeutic implications. Obvious caution should be used in interpretation of transfection experiments using cells that respond to bacterial DNA. Nonspecific immune activation is also documented in experiments involving antisense oligonucleotides (4). While immune activation may be desirable in DNA immunization, it may be a complicating factor in gene therapy and antisense therapy. Immune stimulation by DNA containing unmethylated CpG may explain a number of biologic observations. CpG suppression is thought to be caused by the deamination of 5-methylcytosine and a failure in DNA repair, leaving TpG dinucleotides (7). The fact that mammals have not developed a more efficient mechanism to prevent this potentially harmful mutagenesis suggests that CpG suppression may be desirable. Evolutionary pressure for lowering of CpG levels may have been driven by the development of a means to distinguish between mammalian and foreign DNA (4). Pathogenic organisms may then also be subject to a selective pressure to lower CpG and evade immune responses, and indeed, CpG suppression is observed in a large number of viruses (7).

In the normal in vivo situation, immune activation by DNA could occur either by extracellular DNA from dead organisms, by bacterial DNA released by cell killing in phagolysosomes, or by invading viral DNA. A recognition system for the DNA, and a mechanism for subsequent activation of transcription factors such as NF-κB, remain to be established.

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Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs

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ABSTRACT **Unmethylated CpG dinucleotides in particular base contexts (CpG-S motifs) are relatively common in bacterial DNA but are rare in vertebrate DNA. B cells and monocytes have the ability to detect such CpG-S motifs that trigger innate immune defenses with production of Th1-like cytokines. Despite comparable levels of unmethylated CpG dinucleotides, DNA from serotype 12 adenovirus is immune-stimulatory, but serotype 2 is nonstimulatory and can even inhibit activation by bacterial DNA. In type 12 genomes, the distribution of CpG-flanking bases is similar to that predicted by chance. However, in type 2 adenoviral DNA the immune stimulatory CpG-S motifs are outnumbered by a 15- to 30-fold excess of CpG dinucleotides in clusters of direct repeats or with a C on the 5′ side or a G on the 3′ side. Synthetic oligodeoxynucleotides containing these putative neutralizing (CpG-N) motifs block immune activation by CpG-S motifs *in vitro* and *in vivo*. Eliminating 52 of the 134 CpG-N motifs present in a DNA vaccine markedly enhanced its Th1-like function *in vivo*, which was increased further by the addition of CpG-S motifs. Thus, depending on the CpG motif, prokaryotic DNA can be either immune-stimulatory or neutralizing. These results have important implications for understanding microbial pathogenesis and molecular evolution and for the clinical development of DNA vaccines and gene therapy vectors.**

The genomic DNAs of bacteria and vertebrates differ in the frequency and methylation of CpG dinucleotides, which are relatively common in bacterial DNA (approximately 1/16 bases), but are underrepresented ("CpG suppression"; 1/50–1/60 bases) and methylated in vertebrate DNA (1). Bacterial DNA or synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG dinucleotides in particular base contexts induce B cell proliferation, interleukin (IL)-6 and Ig secretion, and apoptosis resistance (2–12, 16). These immunostimulatory CpGs typically are preceded on the 5′ side by an ApA, GpA, or GpT dinucleotide and followed on the 3′ side by two pyrimidines, especially TpT (CpG-S motif) (11). Monocytes are directly activated by CpG-S motifs to secrete the Th1-like cytokine IL-12 and type I interferons (IFN), and natural killer (NK) cells respond with increased lytic activity and IFN-γ secretion, enhancing protective immune responses (4, 5, 7, 8, 13, 14). Methylated bacterial DNA or ODN in which the cytosines of CpG have been converted to 5-methyl-cytosine (the form present in vertebrate DNA) fails to induce immune activation (2). The stimulatory effects of CpG-S motifs appear to be mediated at least in part by the activation of NF-κB (8, 12, 15, 55). Thus, this simple structural difference in the frequency of CpG-S motifs between vertebrate and prokaryotic genomic DNAs appears to function as a "danger signal" to trigger innate immune defenses against infection and initiate a specific immune response (reviewed in ref. 17). Indeed, ODN containing CpG-S motifs can be mixed with antigens to promote strong Th1-like immune responses (18–23). Recent studies have suggested that effective DNA vaccines require CpG-S motifs within the plasmid backbone (24, 25).

Nearly all DNA viruses and retroviruses appear to have evolved to avoid this defense mechanism through reducing their genomic content of CpG dinucleotides by 50–94% from that expected based on random base usage (26). CpG suppression is absent from bacteriophage, indicating that it is not an inevitable result of having a small genome (26). Statistical analysis indicates that the CpG suppression in lentiviruses is an evolutionary adaptation to replication in a eukaryotic host (27). Adenoviruses, however, are an exception to this rule as they have the expected level of genomic CpG dinucleotides (26). Different groups of adenovirae can have quite different clinical characteristics. Serotype 2 and 5 adenoviruses (subgenus C) are endemic causes of upper respiratory infections and are notable for their ability to establish persistent infections in lymphocytes (28). These adenoviral serotypes are modified frequently by deletion of early genes for use in gene therapy applications, where a major clinical problem has been the frequent inflammatory immune responses to the viral particles. Serotype 12 adenovirus (subgenus A) does not establish latency, but can be oncogenic. Since viruses have evolved a broad range of sophisticated strategies for avoiding or co-opting host immune defenses, we investigated the immune effects of the DNA from these adenoviral serotypes.

METHODS

ODN and DNA. Phosphodiester ODN were purchased from Operon Technologies (Alameda, CA), and nuclease-resistant phosphorothioate ODN were purchased from Oligos Etc. (Wilsonville, OR) or Hybridon Specialty Products (Milford, MA). All ODN had undetectable endotoxin levels (less than 1 ng/mg) by *Limulus* assay (BioWhittaker). *Escherichia coli* (strain B) DNA was purchased from Sigma, purified by repeated extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and/or Triton X-114 extraction and ethanol precipitation, and made single-stranded by boiling for 10 min followed by cooling on ice for 5 min. Digestion with restriction enzymes did not reduce the stimulatory effects of the DNA

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Abbreviations: IL, interleukin; IFN, interferon; ODN, oligodeoxynucleotides; CTL, cytotoxic T lymphocytes; PBMC, peripheral blood mononuclear cells; HBsAg, hepatitis B surface antigen.

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(not shown). Highly purified type 2, 5, and 12 adenoviral DNA was prepared from viral preparations by using standard techniques and processed in the same manner as the *E. coli* DNA. Plasmids for DNA vaccination were purified by using two rounds of passage over Endo-free columns (Qiagen).

Cell Cultures and ELISA Assays for Cytokines. ELISA assays were performed by using standard techniques and commercially available reagents as described previously (3, 11, 29). Standard deviations of the triplicate wells were <10%.

Construction of Optimized DNA Vectors. The starting material was pUK21-A2, an expression vector containing the immediate early promoter of human cytomegalovirus (CMV IE), the bovine growth hormone (BGH) polyadenylation signal, and the kanamycin resistance gene (T.W. and H.D., unpublished data). To avoid disrupting the plasmid origin of replication, mutagenesis designed to eliminate CpG-N motifs was restricted to the kanamycin resistance gene and nonessential DNA sequences after the gene. A total of 22 point mutations were introduced to alter 15 CpG-N motifs (a "motif" refers to a hexamer containing one or more CpG dinucleotides) containing 19 CpG dinucleotides, 12 of which were eliminated and 7 of which were transformed into CpG-S motifs. Site-directed mutagenesis was performed by overlap-extension PCR as described by Ge and coworkers (30). The 1.3-kb *Alw*NI-*Eco*O109 I fragment of pUK21-A2, which contained all 22 nt to be mutated, was used as the template for PCR. The 1.3-kb fragment was regenerated by four rounds of overlap-extension PCR by using appropriate mutagenic primers and substituted for the original *Alw*NI-*Eco*O109 I fragment, resulting in pUK21-B2. All the mutations were confirmed by sequencing.

Another 37 CpG-N motifs were removed by replacing the F1 origin with a multiple cloning site. Oligonucleotides 5′-GCCCTATTTTAAATTCGAAAGTACTGGACCTGTTAACA-3′ and its complementary strand 5′-CGTGTTAACAGGTCCAGTACTTTCGAATTTAAAATAG-3′ were synthesized, and 5′-phosphorylated. Annealing of these two phosphorylated ODN resulted in a 35-bp double-stranded DNA fragment containing four unique restriction enzyme sites (*Dra*I, *Sca*I, *Ava*II, *Hpa*I) and two sticky ends. Replacing the 0.6-kb *Nar*I-*Eco*O109 I fragment of pUK21-B2, which contained the entire F1 ori, with this double-stranded DNA fragment resulted in the master vector pMAS.

Next, different numbers of CpG-S motifs were inserted into the vector by allowing self-ligation of a 20-bp DNA fragment with the sequence 5′-GACTCCATGACGTTCCTGACGTTTCCATGACGTTCCTGACGTTG-3′ with a complementary strand and inserting different numbers of copies into the *Ava*II site of pMAS. Recombinant clones were screened and the two vectors were chosen for further testing with 16 and 50 CpG-S motifs, and named pMCG16 and pMCG50, respectively.

To create a DNA vaccine, the S gene encoding *ay* subtype of hepatitis B surface antigen (HBsAg) was amplified by PCR and cloned into the *Eco*RV-*Pst*I sites of the vectors, resulting in pUK-S, pMAS-S, pMCG16-S, and pMCG50-S, respectively. Vector sequences were confirmed by sequencing and have been deposited in GenBank under accession numbers AFO53406 (pUK-S), AFO53407 (pMAS-S), AFO53408 (pMCG16-S), and AFO53409 (pMCG50-S).

Immunization of Mice Against HBsAg. Immunization of 6- to 8-week-old female BALB/c mice (Charles River Breeding Laboratories) was done by injection into the tibialis anterior muscle (TA) of 1 μg recombinant HBsAg or 10 μg HBsAg-expressing DNA vaccine (31). Assay for antibodies against HBsAg (anti-HBs) was by endpoint dilution and for cytotoxic T lymphocytes (CTL) was by chromium release assay as described previously (19). Both the protein (±ODN) and DNA vaccines were resuspended in saline for injection.

RESULTS

Type 12 Adenoviral DNA Is Immune-Stimulatory, but Types 2 and 5 Adenoviral DNA Are Immune-Neutralizing. To investigate possible functional differences in the immune effects of various prokaryotic DNAs, we determined their ability to induce cytokine secretion from human peripheral blood mononuclear cells (PBMC). In contrast to bacterial DNA and genomic DNA from type 12 adenovirus, DNA from types 2 and 5 adenovirus failed to induce cytokine production (Table 1). In fact, despite their similar frequency of CpG dinucleotides, type 2 or 5 adenoviral DNA severely reduced the cytokine expression induced by coadministered immunostimulatory *E. coli* genomic DNA (Table 2). This indicates that type 2 and 5 adenoviral DNA does not simply lack CpG-S motifs, but contains sequences that actively suppress those in *E. coli* DNA.

Identification of Putative Immune Neutralizing CpG-N Motifs in Types 2 and 5 Adenoviral Genomes. To identify possible nonrandom skewing of the bases flanking the CpG dinucleotides in the various adenoviral genomes, we examined their frequency of all 4,096 hexamers. The six most common hexamers in the type 2 adenoviral genome are shown in Table 3, along with their frequency in the type 12 and *E. coli* genomes. Remarkably, all of these overrepresented hexamers contain either direct repeats of CpG dinucleotides or CpGs that are preceded by a C and/or followed by a G. These CpG-N motifs are approximately 3- to 6-fold more common in the immune inhibitory types 2 and 5 adenoviral genomes than in those of immune-stimulatory type 12 adenoviral, *E. coli*, or nonstimulatory human genomic DNAs (Table 3). This hexamer analysis further revealed that the frequency of hexamers containing CpG-S motifs (e.g., GACGTT or AACGTT) in the type 2 adenoviral genome is as low as that in the human genome: only 1/3 to 1/6 of that in *E. coli* and type 12 adenoviral DNA (Table 3).

Effect of CpG-N Motifs on the Immune Stimulatory Effects of CpG-S Motifs. To determine whether these overrepresented CpG-N motifs could explain the neutralizing properties of types 2 and 5 adenoviral DNA, we tested the *in vitro* immune effects of synthetic oligodeoxynucleotides bearing a CpG-S motif, one or more CpG-N motifs, or combinations of both. An ODN containing a single CpG-S motif induces spleen cell production of IL-6, IL-12, and IFN-γ (ODN 1619, Table 4). However, when the 3′ end of this ODN was modified by substituting either repeating CpG dinucleotides or a CpG dinucleotide preceded by a C, the level of cytokine production was reduced by approximately 50% (ODN 1952 and 1953, Table 4). ODN consisting exclusively of these neutralizing CpG (CpG-N) motifs induced little or no cytokine production (Table 5). Indeed, addition of ODN containing one or more CpG-N motifs to spleen cells along with the CpG-S ODN 1619 caused a substantial decrease in the induction of IL-12 expression, indicating that the neutralizing effects can be exerted in trans (Table 5).

Table 1. Genomic DNA from type 12 but not type 2 adenovirus stimulates cytokine secretion from human PBMC

	Experiment 1		Experiment 2	
DNA added	TNF-α	IL-6	TNF-α	IL-6
Cells	27	800	30	800
E. coli (3 μg/ml)	235	26,500	563	34,000
CT (10 μg/ml)	0	1,400	0	2,800
Adv 2 (3 μg/ml)	15.6	900	30	1,900
Adv 12 (3 μg/ml)	86	11,300	120	11,250

PBMC were obtained from normal human donors and cultured at 1×10^5 cells/200 μl in RPMI 1640 medium with 10% autologous serum for 4 hr (TNF-α assay) or 24 hr (IL-6 assay). The level of cytokine present in culture supernatants was determined by ELISA (pg/ml). Adv, adenovirus serotype.

Table 2. Type 5 adenoviral DNA suppresses the human PBMC cytokine response to *E. coli* (EC) DNA

DNA added	IFN-γ
EC (50 μg/ml)	509
EC (5 μg/ml)	554
EC (0.5 μg/ml)	285
EC (0.05 μg/ml)	173
Adv* (50 μg/ml)	<10
Adv (5 μg/ml)	<10
EC/Adv (50:50 μg/ml)	23
EC/Adv (5:50 μg/ml)	<10
EC/Adv (0.5:0.5 μg/ml)	25

The second column represents the level of IFN-γ production by ELISA (pg/ml) from 24-hr supernatants of human PBMC cultured at 1×10^5 cells/200 μl in RPMI 1640 medium with 10% autologous serum. Similar inhibitory effects were seen when using PBMC from several different donors and from murine spleen cells.
*Type 2 adenoviral DNA had indistinguishable immune effects (not shown).

To determine whether the *in vivo* immune activation by ODN containing CpG-S motifs would be reversed by CpG-N motifs, we immunized mice with recombinant HBsAg, with or without nuclease-resistant phosphorothioate-modified ODN containing various types of CpG motifs. As expected, a CpG-S ODN promoted a high titer of antibodies against HBsAg (anti-HBs antibodies) that were predominantly of the IgG2a subclass, indicating a Th1-type immune response (Fig. 1; ODN 1826). The various CpG-N ODN induced either little or no production of anti-HBs antibodies (ODN 1631, 1984, and 2010) (Fig. 1). Mice immunized with combinations of CpG-S and CpG-N ODN had a reduced level of anti-HBs antibodies compared with mice immunized with CpG-S ODN alone, but these were still predominantly IgG2a (Fig. 1).

Enhanced DNA Vaccination by Deletion of Plasmid CpG-N Motifs. DNA vaccines can be highly effective inducers of Th1-like immune responses (32, 33). Based on the *in vivo* and *in vitro* effects of CpG-N motifs, we hypothesized that their presence within a DNA vaccine would decrease its immunostimulatory effects. Our starting vector, pUK21-A2, contained 254 CpG dinucleotides, of which 134 were within CpG-N motifs. To test the hypothesis that these CpG-N motifs adversely affected the efficacy of this vector for DNA-based vaccination, the number of CpG-N motifs was reduced, either by mutation or deletion. Since mutations in the plasmid origin of replication interfere with replication of the plasmid, we restricted our initial mutations to the kanamycin resistance gene and a nonessential flanking region. We were able to eliminate 19 CpG dinucleotides contained within 15 of the 20

Table 3. Genomic frequencies of selected hexamers

Hexamer	Adenovirus type 2	Adenovirus type 12	*E. coli*	Human
GCGCGC	1.614	0.498	0.462	0.153
GCGGCG	1.530	0.469	0.745	0.285
GGCGGC	1.419	0.440	0.674	0.388
CGCGCG	1.336	0.322	0.379	0.106
GCCGCC	1.280	0.410	0.466	0.377
CGCCGC	1.252	0.410	0.623	0.274
GACGTT (CpG-S)	0.083	0.234	0.263	0.068
AACGTT (CpG-S)	0.056	0.205	0.347	0.056

The frequencies of hexamers in adenoviral and *E. coli* genomes were kindly provided by J. Han (University of Alabama, Birmingham), who also determined those for the human genome (54). The hexamer frequencies in type 5 adenovirus are essentially identical to those in type 2 and therefore are not shown. The last two hexamers are CpG-S motifs shown for comparison and are the most stimulatory of all tested CpG-S motifs (11). Note that the expected frequency of a randomly selected hexamer is $1/4{,}096 = 0.244 \times 10^{-3}$.

Table 4. Identification of neutralizing CpG motifs that reduce the induction of cytokine secretion by a CpG-S motif in the same ODN (cis-neutralization)

ODN	Sequence 5′-3′	ODN-induced cytokine expression IL-6	IL-12	IFN-γ
None		<5	206	898
1619	TCCATGTCGTTCCTGATGCT	1,405	3,130	4,628
1952	. . . . . GCGCGCG	559	1,615	2,135
1953	. . . . . CC. . .	577	1,854	2,000

Dots in the sequence of ODN 1952 and 1953 indicate identity to ODN 1619; CpG dinucleotides are underlined for clarity. ODN without CpG-N or CpG-S motifs had little or no effect on cytokine production. The data shown are representative of four experiments. All cytokines are given in pg/ml and were measured by ELISA on supernatants from DBA/2 spleen cells cultured in 96-well plates at 2×10^7 cells/ml for 24 hr with the indicated ODN at 30 μg/ml. SD of the triplicate wells was <7%. None of the ODN induced significant amounts of IL-5.

CpG-N motifs in these regions without changing the protein sequence. The F1 origin of replication containing 37 CpG-N motifs and only 17 other CpG dinucleotides then was deleted, creating the vector pMAS. This vector was modified further by the introduction of 16 or 50 CpG-S motifs, yielding vectors pMCG16 and pMCG50, respectively. The S gene for HBsAg then was cloned into these vectors downstream from the CMV promoter, to make pUK-S, pMAS-S, pMCG16-S, and pMCG50-S, respectively.

When tested for their ability to induce cytokine (IL-6 and IL-12) secretion from cultured spleen cells, we found that the pMAS-S, pMCG16-S, and pMCG50-S vectors had significantly enhanced immune stimulatory activity compared with pUK-S (not shown). When used as a DNA vaccine, the anti-HBs response at 4 and 6 weeks was substantially stronger with DNA vaccines from which CpG-N motifs had been deleted and even more so when 16 CpG-S motifs had been inserted. The vector with 50 CpG-S motifs, however, was less effective at inducing antibody production than that with 16 motifs (Fig. 2*A*). Removal of CpG-N motifs and addition of CpG-S motifs resulted in a more than 3-fold increase in the proportion of IgG2a relative to IgG1 anti-HBs antibodies, indicating an enhanced Th-1 response. This accentuated Th1 response also was demonstrated by the striking progressive increases in CTL responses induced by vectors from which CpG-N motifs were deleted and/or CpG-S motifs added (Fig. 2*B*).

DISCUSSION

The discovery of immune-activating CpG-S motifs in bacterial DNA has led to the realization that aside from encoding genetic information, DNA can also function as a signal-transducing molecule. Our present results demonstrate that genomic DNA from type 12 adenovirus is immune-stimulatory, compatible with its relatively high content of CpG-S motifs. In contrast, genomic DNA from types 2 and 5 adenoviruses is not stimulatory, but rather is immune-neutralizing and blocks the cytokine induction of bacterial DNA (Tables 1 and 2). To identify possible differences in the CpG motifs present in these different adenoviral genomes, we analyzed the genomic frequency of all hexamer sequences. This analysis demonstrated that only the types 2 and 5 adenoviral genomes had a dramatic overrepresentation of CpG motifs containing direct repeats of CpG dinucleotides and/or CpGs preceded by a C and/or followed by a G (Table 3). Synthetic ODN containing such putative immune-neutralizing (CpG-N) motifs not only did not induce cytokine production *in vitro*, but also inhibited the ability of an immune-stimulatory CpG-S motif to induce cytokine expression (Tables 4 and 5).

Table 5. Inhibition of CpG-induced cytokine secretion by ODN containing CpG-N motifs

CpG-N ODN	Sequence 5′-3′	IL-12 secretion*	CpG-S-induced IL-12 secretion†
None		268	5,453
1895	GCGCGCGCGCGCGCGCGCGC	123	2,719
1896	CCGGCCGGCCGGCCGGCCGG	292	2,740
1955	GCGGCGGGCGGCGCGCGCCC	270	2,539
2037	TCCATGCCGTTCCTGCCGTT	423	2,847

*To detect any effect of CpG-N ODN on basal IL-12 production, BALB/c spleen cells were cultured in 96-well plates at 2×10^7 cells/ml with the indicated ODN for 24 hr and then the supernatants were assayed for IL-12 by ELISA (pg/ml).

†To determine whether CpG-N ODN can inhibit induced IL-12 secretion, cells were set up the same as in * except that IL-12 secretion was induced by the addition of the CpG-S ODN 1619 (TCCATGACGTTCCTGATGCT) at 30 μg/ml. The data shown are representative of five experiments.

These studies reveal that there are immune-neutralizing CpG-N as well as stimulatory CpG-S motifs and that there is a surprisingly complex role for the bases flanking CpG dinucleotides in determining these immune effects. In general, CpG-N motifs oppose CpG-S motifs in cis or trans. Some of the most effective CpG-N ODN are self-complementary and/or G-rich, which may give them the capacity to form higher-ordered structures. Further studies are underway to determine the molecular mechanisms through which CpG-N and CpG-S motifs exert their respective immune effects.

Remarkably, the hexamers that contain CpG-N motifs are from 15 to 30 times more common in types 2 and 5 adenoviral genomes than those that contain immune-stimulatory CpG-S motifs. However, in type 12 adenoviral genomes the frequencies of hexamers containing CpG-N and CpG-S motifs do not differ substantially from chance. These data suggest that the immune-neutralizing effects of types 2 and 5 adenoviral DNA are not merely a result of their propagation in eukaryotic cells, but rather are due to the overall excess of CpG-N compared with CpG-S motifs. It is tempting to speculate that the marked overrepresentation of CpG-N motifs in the genomes of types 2 and 5 adenovirus may contribute to the biologic properties, such as persistent infection of lymphocytes, which distinguish them from type 12 adenovirus. The presence of large numbers of CpG-N motifs within these adenoviral genomes may have played an important role in the evolution of this virus by enabling it to avoid triggering CpG-induced immune defenses. It will be interesting to determine the general distribution of CpG-N and CpG-S motifs in different families of microbial and viral genomes, and to explore their possible roles in disease pathogenesis.

CpG-N motifs also are overrepresented in the human genome, where their hexamers are approximately 2- to 5-fold more common than CpG-S motifs. While this skewing is far less marked than that in adenoviral DNA, it would still be expected to reduce or eliminate any immune-stimulatory effect from the unmethylated CpGs present in CpG islands within vertebrate DNA. We and others have found that even when predominantly or completely unmethylated, vertebrate DNA still is not immune-stimulatory (A.M.K. and P. Jones,

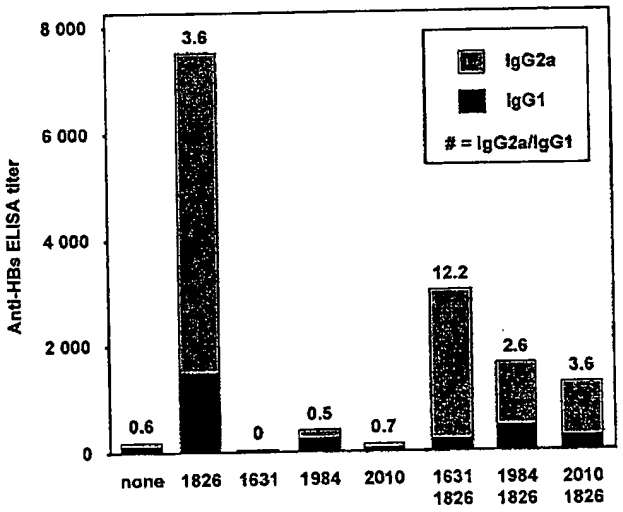

FIG. 1. Induction of a Th2-like response by a CpG-N motif, and inhibition of the Th1-like response induced by a CpG-S motif. Anti-HBs antibody titers (IgG1 and IgG2a subclasses) in BALB/c mice 12 weeks after i.m. immunization with recombinant HBsAg, which was given alone (none) or with 10 μg stimulatory ODN (1826), 10 μg of neutralizing ODN (1631, CGCGCGCGCGCGCGCGCGCG; 1984, TCCATGCCGTTCCTGCCGTT; or 2010, GCGGCGGGCGGCGCGCGCCC; CpG dinucleotides are underlined for clarity) or with 10 μg stimulatory ODN + 10 μg neutralizing ODN. To improve nuclease resistance for these *in vivo* experiments, all ODN were phosphorothioate-modified. Each bar represents the group mean (n = 10 for none; n = 15 for 1826 and n = 5 for all other groups) for anti-HBs antibody titers as determined by end-point dilution ELISA assay. Solid portions of bars indicate antibodies of IgG1 subclass (Th2-like), and shaded portions indicate IgG2a subclass (Th1-like). The numbers above each bar indicate the IgG2a/IgG1 ratio in which a ratio >1 than indicates a predominantly Th1-like response and a ratio <1 indicates a predominantly Th2-like response (a value of 0 indicates a complete absence of IgG2a antibodies).

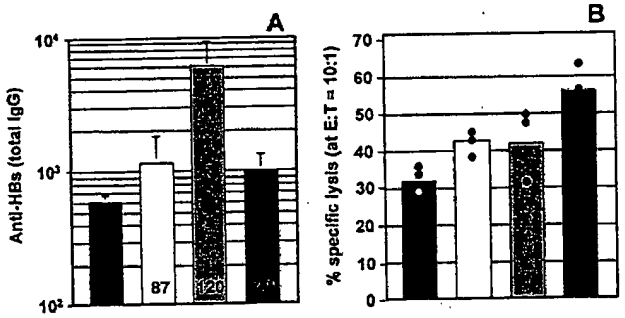

FIG. 2. Enhancement of *in vivo* immune effects with optimized DNA vaccines. Mice were injected with 10 μg of pUK-S (solid bars), pMAS-S (open bars), pMCG16-S (lightly shaded bars), or pMCG50-S (darkly shaded bars) plasmid DNA bilaterally (50 μl at 0.1 mg/ml in saline) into the TA muscle as described (53). (*A*) The anti-HBs antibody response at 6 weeks (detected as described in *Methods*). Bars represent the group means (n = 5) for ELISA end-point dilution titers (performed in triplicate), and vertical lines represent the SEM. The numbers on the bars indicate the ratio of IgG2a/IgG1 antibodies at 4 weeks, as determined in separate assays (also in triplicate) by using pooled plasma. (*B*) CTL activity in specifically restimulated (5 days) splenocytes taken from mice 8 weeks after DNA immunization. Bars represent the group means (n = 3) for percentage of specific lysis (performed in triplicate) at an effector-to-target (E/T) ratio of 10:1; dots represent the individual values. Nonspecific lytic activity determined with non-antigen-presenting target cells, which never exceeds 10%, has been subtracted from values with HBsAg-expressing target cells to obtain percentage of specific lysis values.

unpublished data; ref. 34), which is in keeping with its predominance of CpG-N motifs (Table 3). Given the overall level of CpG suppression in the human genome, the molecular mechanisms responsible for the skewing of the frequency of CpG-N to CpG-S motifs are unclear. Such a distortion from the expected random patterns would seem to require the existence of pathways that preferentially mutate the flanking bases of CpG-S motifs in vertebrate genomes, but do not affect CpG-N motifs. Indeed, statistical analyses of vertebrate genomes have provided evidence that CpGs flanked by A or T (as in CpG-S motifs) mutate at a faster rate than CpGs flanked by C or G (35).

Based on our *in vitro* experiments we hypothesized that the presence of CpG-N motifs in DNA vaccines interferes with the induction of the desired immune response. Indeed, the present study demonstrates that elimination of CpG-N motifs from a DNA vaccine leads to improved induction of antibodies. By removing 52 of the CpG-N motifs from a DNA vaccine (45 were deleted and 7 turned into CpG-S motifs) the serologic response was more than doubled; by then adding an additional 16 CpG-S motifs, the response was enhanced nearly 10-fold (Fig. 2*A*). Likewise, CTL responses were improved by removing CpG-N motifs and even more so by adding 16 or 50 CpG-S motifs (Fig. 2*B*). These increased responses are especially notable in view of the fact that the total number of CpG dinucleotides in the mutated vaccines is considerably below the original number.

The finding that the vector with 50 CpG-S motifs was inferior to that with 16 motifs for induction of humoral immunity was unexpected and may be secondary to CpG-induced production of type I interferons and subsequent reduction in the amount of antigen expressed. The decreased antibody response induced by pMCG50-S seems unlikely to be explained by vector instability since this vector gave the best CTL responses (Fig. 2*B*). Although the pMCG50-S vector was slightly larger than pMCG16-S, the 10-μg dose still contained 93% as many plasmid copies as it did pMCG16-S, so lower copy number is unlikely to account for the reduced antibody levels. The current generation of DNA vaccines are quite effective in mice, but much less effective in primates (36–42). Our present results suggest that attaining the full clinical potential of DNA vaccines may require using engineered vectors in which CpG-N motifs have been deleted and CpG-S motifs have been added.

On the other hand, the field of gene therapy may benefit from the discovery of CpG-N motifs through their insertion into gene transfer vectors to prevent or reduce the induction of host immune responses. Most of the CpG-N motifs in the adenoviral genome are in the left-hand (5′) side, which generally is partially or totally deleted for the preparation of gene therapy vectors, especially with the "gutless" vectors (43). This could lead to an enhanced CpG-S effect. Since nucleic acids produced in viral vectors are unmethylated, they may produce inflammatory effects if they contain a relative excess of CpG-S over CpG-N motifs and are delivered at an effective concentration (about 1 μg/ml). Gene therapy studies with adenoviral vectors have used doses up to 10^{10} infectious units (IU)/ml (which contains 0.4 μg of DNA/ml based on the genome size of 36 kb). Given that approximately 99% of adenoviral particles are noninfectious, this corresponds to a DNA dose of approximately 40 μg/ml, which is well within the range at which CpG DNA causes *in vivo* immune-stimulatory effects; just 10 μg/mouse induces IFN-γ production (5), acts as an adjuvant for immunization (19, 20–23), and causes acute pulmonary inflammation when delivered into mouse airways (44). Multiple mechanisms besides the presence of CpG-S DNA doubtless are responsible for the inflammatory responses that have limited the therapeutic development of adenoviral vectors (45, 46). Nonetheless, our present results suggest that consideration be given to the maintenance or insertion of CpG-N motifs in adenoviral vectors and to the engineering of inserts so that CpG-S motifs are mutated to reduce immune activation.

In recent years, it has become clear that effective gene expression need not require a viral delivery system. The use of plasmids for gene delivery (with or without lipids or other formulations) avoids some of the problems of viral vectors. On the other hand, much larger doses of DNA typically are required, since delivery is far less efficient than with a targeted system such as a virus. For example, effective gene expression in mice typically may require 500–1,000 μg DNA/mouse (47, 48). A recent human clinical trial using lipid/DNA complexes and naked DNA for delivery of cystic fibrosis transmembrane conductance regulator to the nasal epithelium of patients with cystic fibrosis used doses of 1.25 mg of plasmid/nostril (49). The successful application of naked DNA expression vectors for gene therapy will depend on the safety of repeatedly delivering high doses of DNA. Since the plasmids used for gene therapy typically contain several hundred unmethylated CpG dinucleotides, many of which are in CpG-S motifs, some immune activation may be expected to occur. Indeed, mice given repeated doses of just 10 μg of plasmid DNA daily develop elevated lymphocyte levels (50), and several humans who received intranasal plasmid DNA had elevated serum IL-6 levels (47). Furthermore, delivery of 4 mg of a gene therapy plasmid to cystic fibrosis patients in a recent clinical trial caused acute onset of symptoms compatible with immune activation, including fever, chills, and pulmonary congestion (J. Zabner, personal communication). Another reason to avoid the presence of CpG-S motifs in gene therapy vectors is that the cytokines that are produced because of the immune stimulation may reduce plasmid vector expression, especially when this is driven by viral promoters (32).

It is, therefore, highly desirable to develop improved gene delivery systems with reduced immune activation. It is not possible to simply methylate the CpG-S dinucleotides in gene therapy plasmids, since methylation of promoters abolishes or severely reduces their activity (29, 49). The only promoter resistant to methylation-induced silencing is the mouse mammary tumor virus promoter, which contains no essential CpGs, but is fairly weak (51). In any case, even when the promoter is unmethylated, expression is still greatly reduced if the coding sequences are methylated (50). In fact, even the strong CMV IE promoter is inactivated completely by CpG methylation (51). Deletion of all CpGs from an expression plasmid is not feasible since many of these are located in the origin of replication (approximately 1.2 kb long), where even single base changes can dramatically reduce plasmid replication. For these reasons, we propose that the addition of CpG-N motifs and/or mutation or conversion of CpG-S to CpG-N motifs may lead to the generation of less immune-stimulatory vectors for gene therapy. Studies to investigate this possibility are underway.

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Brief Definitive Report

DNA as an Adjuvant: Capacity of Insect DNA and Synthetic Oligodeoxynucleotides to Augment T Cell Responses to Specific Antigen

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Summary

How strong adjuvants such as complete Freund's adjuvant (CFA) promote T cell priming to protein antigens in vivo is still unclear. Since the unmethylated CpG motifs in DNA of bacteria and other nonvertebrates are stimulatory for B cells and antigen-presenting cells, the strong adjuvanticity of CFA could be attributed, at least in part, to the presence of dead bacteria, i.e., a source of stimulatory DNA. In support of this possibility, evidence is presented that insect DNA in mineral oil has even stronger adjuvant activity than CFA by a number of parameters. Synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs mimic the effects of insect DNA and, even in soluble form, ODNs markedly potentiate clonal expansion of T cell receptor transgenic T cells responding to specific peptide.

It is now well established that unmethylated CpG dinucleotide motifs of bacterial DNA have the capacity to cause polyclonal activation of B cells and stimulation of APCs (1–8). The immunostimulatory property of unmethylated CpG motifs is not unique to bacteria and applies to a wide spectrum of nonvertebrates including insects, nematodes, mollusks, and yeast (3, 9, 10); by contrast, DNA from various vertebrates, e.g., frogs and fish, is nonstimulatory. The capacity of nonvertebrate DNA to stimulate B cells and APCs is shared by synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs (5, 11). When coinjected with antigen, these agents also enhance the generation of cytotoxic T cell activity and production of specific antibody and IFN-γ (12–15).

Stimulation of APCs via unmethylated CpG motifs could explain the remarkable efficacy of "naked" DNA vaccines (16). In this respect, the induction of antigen-specific responses after DNA vaccination is reported to be much more efficient when the plasmid vector for mammalian DNA contains unmethylated CpG motifs (17, 18). In light of this finding, DNA vaccines may operate not only by providing a source of specific antigen (peptide) but by acting as an adjuvant, i.e., by enhancing the immunogenicity of APCs. According to this notion, the poor immunogenicity of proteins or peptides given in solution could be overcome simply by coinjecting any source of DNA containing stimulatory CpG motifs. In support of this prediction we show here that, when suspended in mineral oil, insect DNA and ODNs containing unmethylated CpG motifs act as powerful adjuvants in mice when coinjected with foreign peptides or proteins. ODNs also have adjuvant activity in soluble form and markedly amplify clonal expansion of TCR transgenic T cells responding to specific peptide.

Materials and Methods

Mice. C57BL/6J (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). 2C TCR transgenic mice (9) were bred and maintained at The Scripps Research Institute (La Jolla, CA).

Proteins and Peptides. Fowl γ-globulin (FγG) was purchased from Pel-Freez Biologicals (Rogers, AR). A synthetic peptide, SIYRYYGL (19), recognized by 2C TCR transgenic cells in the context of H-2K^b was provided by Z. Cai (R.W. Johnson Pharmaceutical Research Institute, San Diego, CA). This peptide was synthesized on a synthesizer (431 A; Applied Biosystems, Foster City, CA) and purified with C18 reverse-phase high performance liquid chromatography.

DNA and ODNs. DNA from the *Drosophila melanogaster* cell line, SC2, was prepared as described (20). For injection, DNA was used without denaturation. CpG (GCATGACGTTGAGCT) and ZpG (GCATGAZGTTGAGCT, Z = 5′-methyl-C) phosphorothioated ODNs were designed using published sequences (5). The ODNs were synthesized and purified using HPLC by Research Genetics, Inc. (Huntsville, AL). Residual LPS in DNA preparations was measured (Limulus Amebocyte Lysate QCL-1000 kit; BioWhittaker, Walkersville, MD). *D. melanogaster* DNA preparations contained 0–10 pg of LPS/mg of DNA.

Immunization with FγG and antibody production. Mice were injected subcutaneously with FγG ± adjuvant in the lower portion

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of both hind limbs in a volume of 50 μl/limb. For injection, FγG was either (*a*) suspended in saline with soluble insect DNA or ODNs, or (*b*) mixed with CFA or IFA (mineral oil) plus either insect DNA or ODNs; in each situation, the dose of FγG injected was the same. Specific antibody production was determined by coating 96-well flat-bottomed plates with 2 μg FγG/well. Serially diluted antiserum were allowed to bind to the plates, washed, and then detected with isotype-specific biotinylated antibodies and streptavidin–horseradish peroxidase (PharMingen, San Diego, CA).

In Vitro T Proliferation Assay and IFN-γ Production. To measure proliferative responses to FγG, draining (popliteal) LN cells from FγG-primed mice were removed at day 9 after priming and then cultured with the indicated concentration of FγG in 200 μl volumes in 96-well plates using standard tissue culture medium plus FCS (21); in some experiments, B cell–depleted LN cells or purified LN $CD4^+$ cells (21) were used as responders. To measure proliferation, cultures were harvested on day 4 after overnight addition of 1 μCi [^{3}H] thymidine (TdR). To measure IFN-γ production, aliquots of culture supernatants were collected on day 2 or 3; IFN-γ production was measured with an ELISA assay using anti–IFN-γ mAbs from PharMingen.

Adoptive Transfer, Immunization, and In Vivo Proliferation of TCR Transgenic T Cells. For adoptive transfer, doses of 2×10^7 unseparated spleen plus LN cells from 2C mice on a B6 background were injected intravenously into normal B6 mice. The recipients were then injected subcutaneously with peptide ± soluble ODNs in both hind limbs. To measure proliferation in vivo, groups of the recipients were given a single injection of 1 mg bromodeoxyuridine (BrdU) intraperitoneally at 3, 4, or 5 d after immunization; BrdU incorporation was measured 4 h after BrdU injection.

Cell Surface Staining and Flow Cytometry. As described elsewhere (22), cell suspensions were first surface stained for expression of CD8 and the TCR clonotype of 2C cells, detected by 1B2 mAb (23). After fixation, the cells were then stained internally for BrdU incorporation using an anti-BrdU mAb (Becton Dickinson, San Jose, CA). Stained cells were analyzed on a FACScan® flow cytometer.

Results

In previous studies, the effects of SssI methylase treatment indicated that the capacity of insect (*D. melanogaster*) DNA to cause polyclonal activation of B cells was controlled, at least in part, by unmethylated CpG motifs (9). To test whether insect DNA could act as an adjuvant for antigen-specific T cell responses, mice were injected subcutaneously with FγG mixed with insect DNA (100 μg/mouse); since DNA is highly unstable when injected in vivo, some mice received FγG plus insect DNA suspended in IFA. Control mice received FγG in saline or suspended in CFA or in IFA without DNA.

Adjuvant Effect of Insect DNA on Normal $CD4^+$ T Cells. Priming of mice injected with FγG ± adjuvant was measured by removing the draining LN (DrLN) at 9 d after immunization and culturing LN cell suspensions with or without FγG in vitro. The results of culturing either unseparated LN cells or purified $CD4^+$ cells with FγG are shown in Fig. 1 *A*. As expected, for both cell populations, in vitro T proliferative responses to FγG were substantial with in vivo priming to FγG in CFA and somewhat lower with priming in IFA. When mice were primed with FγG in saline alone (not shown) or with FγG plus soluble insect DNA (Fig. 1 *A*), secondary responses to FγG in vitro were virtually undetectable, indicative of minimal immunization. In marked contrast, priming with FγG plus insect DNA suspended in IFA led to strong secondary responses to FγG in vitro. Significantly, these responses were appreciably

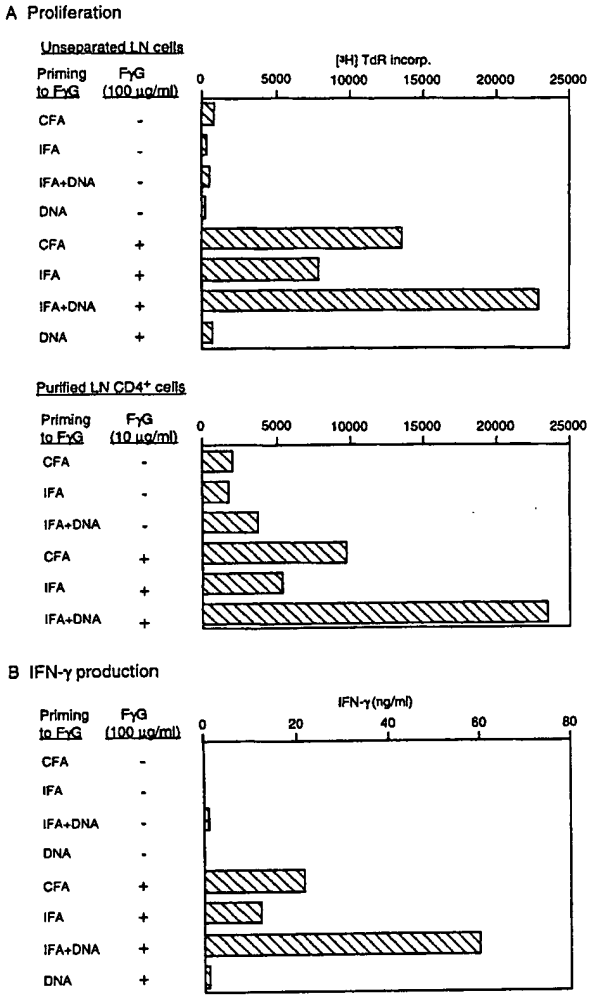

Figure 1. Adjuvant function of insect DNA for T cell responses to FγG. Normal B6 mice were immunized in the hind limbs with FγG (25 μg/limb for *A*, *top* and *B*, 5 μg/limb for *A*, *bottom*) mixed with CFA, IFA, DNA, or IFA and DNA; for DNA, insect DNA was injected at 50 μg/limb. Cell suspensions were prepared from the DrLN 9 d later. For *A*, *top* and *B*, unseparated LN cells were cultured at 2.5×10^5 cells/well ± FγG (20 μg/well, 100 μg/ml). For *A*, *bottom*, purified $CD4^+$ cells were prepared from DrLN cells and cultured at 10^5/well in the presence of T-depleted spleen cells (4×10^5/well) ± FγG (10 μg/ml). To measure [^{3}H]TdR incorporation (*A*), cultures were pulsed with 1 μCi/well of [^{3}H]TdR on day 3 and harvested for radioactive counting on day 4. For IFN-γ production (*B*), supernatants were tested on day 3. The data show means of triplicate cultures; SDs were within 10–20% of the means.

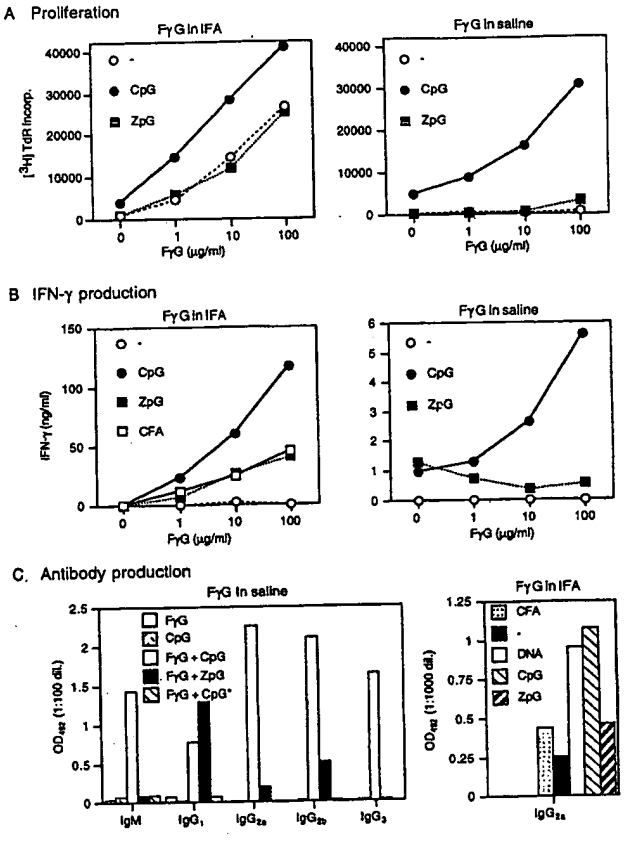

Figure 2. Adjuvant function of ODNs for T cell responses to FγG. Mice were immunized to FγG (5 μg/limb) as described for Fig. 1, using CpG and ZpG ODNs (25 μg/limb) instead of DNA; ODNs were either mixed with IFA (*A*; *B*, *left*; and *C*, *right*) or suspended in saline (*A*; *B*, *right*; and *C*, *left*). Antiserum was collected by tail bleeding on day 9 and tested at 1:100 or 1:1,000 dilution (*C*). For Ab production, some mice received CpG ODNs in the front limbs and FγG in the hind limbs (*FγG + CpG**). For in vitro responses (*A* and *B*), DrLN cells were removed on day 9, depleted of B cells, and cultured with graded doses of FγG for 3 d (IFN-γ production) or 4 d ([3H]TdR incorporation). The data show means of triplicate cultures. Two other experiments gave similar findings.

higher than with priming to FγG in CFA. Similar results occurred for production of IFN-γ in vitro (Fig. 1 *B*) and also for production of specific Ab (see below). As a negative control for insect DNA, we used DNA from nonvertebrates (salmon testes; reference 10). In contrast to insect DNA, salmon DNA in IFA plus FγG was no more immunogenic than IFA plus FγG alone (data not shown).

Adjuvant Effect of Synthetic ODNs on Normal T Cells. The above data indicate that insect DNA acts as a powerful adjuvant, though only when suspended in IFA. To assess whether the adjuvanticity of DNA is controlled by unmethylated CpG motifs, we prepared two synthetic 15-mer ODNs containing a single CG dinucleotide pair. The only difference between the two ODNs was that, for the CG pair, C was unmethylated for one ODN (CpG ODN) but methylated (ZpG ODN) for the other; to retard degradation in vivo, both ODNs contained a phosphorothioated backbone.

To assess adjuvanticity, mice were primed with FγG plus 50 μg/mouse of CpG or ZpG ODNs suspended either in IFA or saline. As measured by secondary T proliferative responses in vitro to graded concentrations of FγG (Fig. 2 *A*, *left*), priming with FγG in IFA was considerably augmented by addition of CpG ODNs; by contrast, addition of ZpG ODNs to IFA had no effect. Thus, for ODNs in IFA, only CpG and not ZpG ODNs had demonstrable adjuvant activity (relative to priming in IFA alone). The results were quite similar for IFN-γ production, except that, for this assay, FγG priming with IFA plus ZpG was clearly higher than with IFA alone (Fig. 2 *B*, *left*). In general, FγG priming with IFA and CpG ODNs was substantially more effective than priming with CFA, especially for IFN-γ production (Fig. 2 *B*, *left*, and data not shown).

The above data refer to ODNs suspended in IFA. Significantly, in contrast to soluble insect DNA, CpG ODNs in saline displayed quite strong adjuvant activity for T proliferative responses (Fig. 2 *A*, *right*); by contrast, ZpG ODNs in saline were ineffective. For IFN-γ production, priming with CpG ODNs in saline led to much lower responses than with CpG ODNs in IFA (Fig. 2 *B*). Nevertheless, IFN-γ production elicited by ODNs in saline was clearly demonstrable with CpG ODNs, but undetectable with ZpG ODNs (Fig. 2 *B*, *right*).

Confirming the results of others (12–15), CpG ODNs acted as a powerful adjuvant for specific Ab production (Fig. 2 *C*). In saline (Fig. 2 *C*, *left*), CpG ODNs augmented both IgM and IgG Ab to FγG; except for IgG_1 Ab, ZpG ODNs were much less effective. Addition of IFA considerably augmented the adjuvant activity of CpG (but not ZpG) ODNs, especially for IgG_{2a} (Fig. 2 *C*, *right*), IgG_{2b}, and IgG_3 (data not shown) Ab. For these isotypes, Ab production elicited by CpG ODNs in IFA was substantially higher than with CFA immunization (Fig. 2 *C*, *right*); similar findings occurred with insect DNA in IFA (Fig. 2 *C*, *right*). By contrast, CpG ODNs or insect DNA in IFA inhibited the production of IgG_1 Ab, relative to immunization with CFA or IFA alone (data not shown). Significantly, the adjuvant activity of CpG ODNs required coinjection with FγG in the same site. Thus, injection of CpG ODNs in the front limbs and FγG in the hind limbs failed to elicit Ab production (Fig. 2 *C*, *left*).

Adjuvant Effects of ODNs on TCR Transgenic $CD8^+$ Cells. Since adjuvants presumably act largely by augmenting the clonal expansion of antigen-specific T cells, we sought direct evidence on this issue by studying the capacity of ODNs to augment proliferation of TCR transgenic T cells to specific peptide in vivo. For these studies we used 2C TCR transgenic mice. For this line, $CD8^+$ T cells have strong reactivity for a synthetic peptide, SIYRYYGL (19), presented by self (K^b) class I molecules. Using 2C mice on a B6 background, the approach taken (24) was to transfer doses of 2×10^7 2C lymphoid cells (pooled from spleen and LN) intravenously into normal B6 mice and then inject the mice subcutaneously with specific peptide ± CpG or ZpG ODNs in saline. To measure T cell proliferation in vivo, groups of the recipients were injected with the DNA

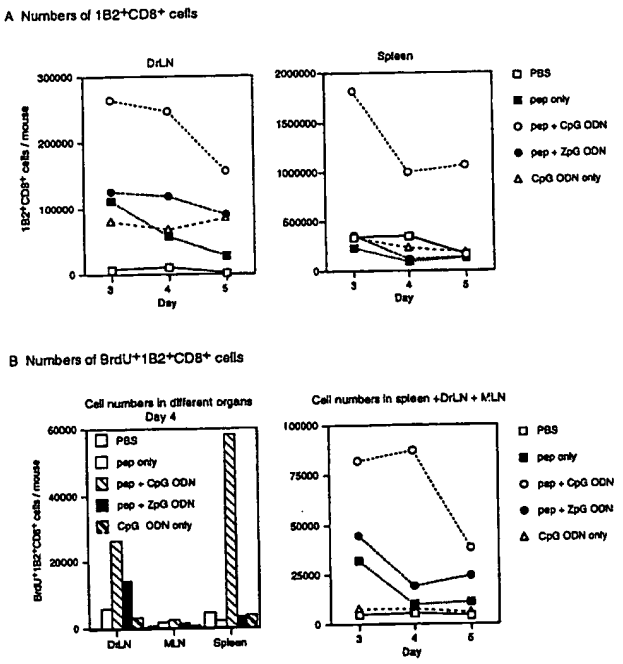

Figure 3. Capacity of ODNs to augment clonal expansion of 2C transgenic $CD8^+$ cells responding to specific peptide. Groups of B6 mice were injected intravenously with 2×10^7 2C lymphoid cells and then injected subcutaneously in the hind limbs with specific peptide (25 μg/limb) ± soluble CpG or ZpG ODNs (25 μg/limb). At 3, 4, or 5 d after immunization, the mice received a single intraperitoneal injection of 1 mg BrdU. Lymphoid organs were removed from the mice 4 h later and cell suspensions were stained for CD8 and 1B2 expression and then for BrdU incorporation followed by FACS® analysis. The data show mean values (2–3 mice/group) for total numbers of $1B2^+$ $CD8^+$ cells (*A*) and $BrdU^+$ $1B2^+$ $CD8^+$ cells (*B*) in the DrLN, spleen, and MLN. Essentially identical results were seen in a second experiment.

precursor, BrdU, at 3, 4, or 5 d after immunization; 4 h after BrdU injection, cell suspensions were stained for surface markers and then, after fixation, for BrdU incorporation. This 4-h pulse approach (22) thus provided an indication of the extent of donor cell proliferation at daily intervals from days 3 through 5. Donor $CD8^+$ cells were detected by staining for expression of CD8 and the 2C TCR clonotype, 1B2.

As shown in Fig. 3 *A*, *left*, total numbers of donor $CD8^+$ cells ($1B2^+$ $CD8^+$ cells) in the DrLN were substantially higher after injection of peptide and CpG ODNs than with injection of peptide alone; ZpG ODNs were much less effective. Similar findings applied to donor cell proliferation, i.e., to total numbers of $BrdU^+$ $1B2^+$ $CD8^+$ cells in the DrLN (Fig. 3 *B*, *left*).

These data refer to the response of donor $CD8^+$ cells in the DrLN. Surprisingly, injecting peptide plus CpG ODNs caused a marked increase in total numbers of both $1B2^+$ $CD8^+$ cells (Fig. 3 *A*, *right*) and $BrdU^+$ $1B2^+$ $CD8^+$ cells (Fig. 3 *B*, *left*) in the spleen, though not in mesenteric LN (MLN). By contrast, injection of peptide alone or peptide plus ZpG ODNs caused little, if any, evidence of proliferation in the spleen.

A rough estimate of the extent of donor T cell proliferation in the whole animal was obtained by calculating total numbers of $BrdU^+$ $1B2^+$ $CD8^+$ cells in DrLN + MLN + spleen at days 3 through 5. By this parameter, priming with peptide plus CpG ODNs was far more effective than priming with peptide alone (Fig. 3 *B*, *right*); priming with peptide plus ZpG ODNs was only slightly better than with peptide alone.

Discussion

Since CFA has long been the "gold standard" for adjuvant function, it is of interest that the adjuvant activity of insect DNA in mineral oil (IFA) surpassed the activity of CFA by three different parameters, namely T proliferative responses, IFN-γ synthesis, and production of specific Ab. This finding supports the view that the adjuvant activity of CFA is due, at least in part, to the presence of dead bacteria, a source of immunostimulatory DNA (3).

It should be emphasized that insect DNA only displayed adjuvant activity when suspended in mineral oil; in soluble form, insect DNA was ineffective, presumably reflecting rapid degradation by enzymes. In view of this problem, we resorted to the use of phosphorothioate-modified synthetic ODNs, which are comparably resistant to degradation in vivo (5). Except for CpG methylation, the two ODNs studied were identical. Confirming the findings of others (5), preliminary data established that CpG ODNs were highly effective in stimulating B cell proliferation in vitro, whereas ZpG ODNs had minimal activity (our unpublished data). Significantly, this marked difference between CpG and ZpG ODNs also applied to adjuvant activity. Thus, unlike ZpG ODNs, CpG ODNs acted as a strong adjuvant when used to prime mice for T proliferative responses, IFN-γ synthesis, and production of specific Ab to FγG. Although the adjuvant activity of CpG ODNs was clearly much higher when suspended in IFA, priming in the presence of soluble CpG ODNs led to quite strong T proliferative responses and low but significant production of specific Ab and IFN-γ. Confirming previous findings (12–15), the adjuvant activity of CpG ODNs for Ab production was much more prominent for certain Ig isotypes, e.g., IgG_{2a}, than for others, notably IgG_1; similar findings applied to insect DNA. Thus, for synthetic ODNs and DNA, the adjuvant function of CpG motifs appears to be skewed to Th1 function (12–15).

Examining the influence of adjuvants during the early primary response is difficult in normal mice, but relatively easy in TCR transgenic mice. When TCR transgenic T cells are exposed to specific peptide on adoptive transfer, it is well established that a mixture of peptide in CFA leads to a prolonged proliferative response (24); by contrast, injection of peptide alone elicits only transient proliferation followed by rapid elimination of the responding T cells. In line with these findings, the response of 2C $CD8^+$ cells to specific peptide alone was very brief and declined abruptly after day 3. By contrast, supplementing peptide with solu-

ble CpG ODNs augmented and considerably prolonged the proliferative response, indicative of an adjuvant effect.

Rather surprisingly, the proliferative response elicited by peptides plus CpG ODNs involved not only the DrLN but also the spleen. Yet proliferation in MLNs was undetectable. How can this distribution be explained? The simplest possibility in our view is that, in contrast to peptide alone, exposure to peptide plus ODNs in the DrLN signaled the responding T cells to survive and make their way into the circulation, thus reaching the spleen. The failure of the cells to reach MLNs may have reflected that antigen activation of T cells often leads to downregulation of the LN homing receptor, CD62L (25), thus preventing migration to LN but not to spleen. In fact, in more recent studies, a high proportion (50%) of the $1B2^+$ $CD8^+$ cells in the spleen on day 4 were $CD62L^{lo}$ (data not shown); such downregulation of CD62L did not apply to MLNs and, in spleen, was only seen with injection of peptide plus CpG ODNs.

How DNA and ODNs potentiate clonal expansion of antigen-specific T cells in vivo is still unclear. Others have postulated that ODNs act directly on T cells and provide a second signal for cells subjected to TCR ligation (12). The alternative possibility is that ODNs function largely by potentiating APC function, e.g., by inducing synthesis of cytokines such as IL-1, TNF-α, and IL-6 (6, 7, 26, 27), thus causing migration of APCs to DrLN (28), and perhaps also by stimulating upregulation of costimulatory molecules on APC precursors, e.g., by IFN-I (15, 29, and our unpublished data). However, direct evidence on the mechanism of action of ODNs under in vivo conditions is still unavailable.

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